Influence of exogenous factors on glucosinolate accumulation in horseradish (*Armoracia rusticana* Gaertn., Mey. & Scherb.)

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Dedicated to:

My Family

Contents

Abbreviations

NAA	1-naphthelen acetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
GN	2-phenylethyl glucosinolate (gluconasturtiin)
SI	2-propenyl glucosinolate (sinigrin)
ABA	Abscisic acid
B	Badish variety
BA	Benzyl adenine
DW	Dry weight
DW%	Dry weight share
E	Eastern variety
ESP	Epithiospecifier proteins
FW%	Fresh weight share
FW	Fresh weight
GS	Glucosinolates
^{o}C	Grad celicius
SB	4-hydroxybenzyl glucosinolate (sinalbin)
IS	Internal standard
K	Krönner variety
MS	Murashige and Skoog medium
MyAP	Myrosinase associated proteins
MBPRP	Myrosinase binding proteins related proteins
MBP	Myrosinase binding proteins
OPA	*o*-phynelphyldehyde
RS	Rain shelter
SA	Salicylic acid
TFP	Thiocyanates forming proteins
Total GS	Total glucosinolates (sum of sinigrin and gluconasturtiin)
JA	Jasmonic acid
MeJA	Methyl jasmonate
ET	Ethylene
GAD	Glutamate decarboxylase
GABA	γ-aminobutyric acid
F	Furrows
R	Ridges, in relation with soil design
R	Variable side chain, in relation with chemical structures
GST	Glutathione-S-transferase
S-GT	S-glucosyltransferase
ST	Sulfotransferase
Glu	Glucose
ESM	Epithiospecifier modifier
ITCs	isothiocyanates
MAM	Methylthioalkylmalate synthase
MeOH	Methanol
ACN	Acetonitrile
RP-HPLC	Reverse phase high performance liquid chromatography

IPC	Ion pair chromatography
US	United States
E.C.	European commission (in relation with enzyme classification)
PEG	Polyethylene glycol
GABA-T	GABA transaminase
SSADH	Succinate semialdehyde dehydogenase
WHC	Water holding capacity
MS	Moderate stress
SS	Severe stress
WE	Excess of water
TBA	Tetrabutylammonium hydrogen sulfate
CaM	Calmodulin

1. Introduction

Plants strongly affect our life and wellbeing by providing food, which contain many important bioactive compounds that influence the nutrition and health (Campbell and Reece, 2004). Apart from cereals, vegetables are of major relevance; they are among the most cultivated crops in the world. In Germany alone more than 120 thousand hectares were grown with vegetables in 2011 compared to about 97 thousand hectares cultivated in the year 2000, which corresponds to ≈25% increase of the cultivated area. At the same time period, yield increased from 3 to 3.7 million tons. Brassicaceae crops composed about 19% of total area grown with vegetables in Germany in 2010, with 0.94 million tons composing about 30% of total vegetables production in the year 2010 (Eurostat, 2012). The Brassicaceae family represents many commercially important crops, such as oilseed rape, broccoli, cabbage, horseradish and cauliflower. These crops are cultivated as human food, animal feed as well as many other agricultural and industrial uses.

Brassicaceae crops have been part of human´s life since a long time. Ancient civilizations used these vegetables as food and medicine; records mentioning these crops are found in Sanskrit, Chinese, Greek, Roman and Arabic. Pythagoras and Hippokrates have described the medicinal properties of mustard oil on 530 BC and 400 BC, respectively. In China, mustard seeds belonging to 4000 BC have been found. Large distribution of these crops cultivation occurred in Europe in the middle ages (Fenwick and Heany, 1983).

Horseradish (*Armoracia rusticana* Gaertn., Mey. & Scherb.) is one of the most important crops of the Brassicaceae family, grown nowadays in Europe and Northern America. In the United States (US) about 1.6 thousand hectares of horseradish are harvested annually (Bratsch, 2009). Unfortunately, there are no specific data available concerning horseradish cultivation and production in Germany.

In addition to their relevance in daily life, Brassicaceae are also important in basic science. *Arabidopsis thaliana* is the most important model plant in molecular biology. Research on *A. thaliana* has greatly enriched our knowledge about biochemical and genetic processes in plants (Halkier and Gershenzon, 2006; Wittstock and Halkier, 2002; Selmar, 2010).

Plants cultivated under arid or semi-arid conditions, as found in the Mediterranean region, produce better spices in terms of taste and aroma intensity than plants cultivated in Central

Europe, characterized by moderate climate. These differences are primarily due to lower contents of relevant plant secondary metabolites, and are usually explained by differences in the exposure of the plants to sunlight, which indeed is highly different between semi-arid and moderate climate conditions. The corresponding coherences are frequently explained by the trivial statement: "the plants in Southern Europe are exposed to much higher rates of sunshine". However, sunlight is not a limiting factor of plant growth, except in extreme situations like constant shadow. Generally, absorbed light energy is higher than plants needs for photosynthetic CO_2-fixation (Wilhelm and Selmar, 2011; Selmar and Kleinwächter, 2013). Furthermore, under high solar irradiation, plants regularly suffer from drought stress, since evaporative demand often is increased by co-occurring elevated temperatures. Major plant responses to stress are characterized by significant changes in the metabolic status, which also should affect the synthesis and accumulation of secondary plant metabolites.

Wilhelm and Selmar (2011) pointed out that due to partial stomata closure caused by drought stress, an over-reduced status results, which affects the biosynthesis rate of secondary plant products. This in fact, would imply that the content of secondary plant products, and thus the quality of spices could be modulated and improved by deliberately applying drought stress. Unfortunately, these coherences have not adequately been addressed so far; thus thorough investigations are required, to get a clear picture of this issue.

1.1. Objectives

The aim of this work is to investigate the effect of exogenous abiotic factors on the concentration and content of glucosinolates in horseradish by exemplarily analyzing the impact of water shortage and high salt concentrations as well as the effect of important signaling compounds. To achieve this goal, corresponding trials with horseradish *in vitro* plants as well as with intact plants had been performed: The *in vitro* studies mainly focused on the impact of stress elicitors (e.g. NaCl and PEG) and the plant stress hormone (abscisic acid), while the trials with the standard horseradish culture varieties dealt with the effects of drought and salt stress as well as with the impact of salicylic acid on glucosinolate synthesis (rain shelter and field trials). The plant materials were analyzed for their concentrations and total contents of glucosinolates (sinigrin and gluconasturtiin). The concentration of the non-proteinogenic amino acid γ-aminobutyric acid (GABA) was determined as a general stress marker. In addition, a brief investigation of drought effect on myrosinase activity was carried out. By this approach, on the one hand the complex interactions between plant stress and

plant secondary metabolism should be elucidated; on the other hand the feasibility to utilize these factors to improve the quality of horseradish tubers in particular and plant derived foods in general should be investigated.

2. Scientific Background

In order to outline the scientific background of this work, a brief overview on the relevant literature is presented in this chapter. Apart from general aspects of horseradish, relevant information on glucosinolates is presented, followed by basic indications of plant stress metabolism.

2.1. Horseradish

Horseradish (*Armoracia rusticana* Gaertn., Mey. & Scherb.) is a perennial plant originating from Eastern Europe and grown in temperate regions all over the world nowadays. It is used fresh or as condiment in mayonnaise and food sauce. Both roots and leaves can be eaten, but commercial importance is due to the pungent root (Courter and Rhodes, 1969; Bratsch, 2009). Moreover, horseradish is used as medicinal plant to cure some ailments, such as coughs, bronchitis, sinus congestion, and urinary tract infections (Ravindran and Pillai, 2004).

2.1.1. Origin

Based on geographical and philological data, horseradish is supposed to be originating from temperate regions of Eastern Europe (Courter and Rhodes, 1969). *Chren,* a common word of Slavic language, is the origin of the names of horseradish in German and French dialects, and all of them mean sea-radish. Other names for horseradish are known in other European civilizations, but *chren* stays the most ancient one. The German word “Meerrettich” was probably the origin of the English name (horseradish). By misunderstanding the German word Meer (sea) as Mähre (old horse), the new name “horse-radish” was developed instead of the original one (sea-radish) (Courter and Rhodes, 1969).

2.1.2. Classification

The word *Armoracia* which is used to describe the horseradish genus is derived from Celtic and means a plant grown near the sea. Although botanists of the 18th century and later listed horseradish under different genera and species, *Armoracia lapathifolia* and *Armoracia rusticana* are the most common nowadays, and most botanists prefer to use *Armoracia rusticana* (Courter and Rhodes, 1969; Weber, 1949).

Many strains of *Armoracia rusticana* are available for commercial production of horseradish, these strains can broadly be divided into two varieties: Common and Bohemian. Due to its high quality large roots, Common is preferred for commercial production. However, this variety is very susceptible for virus diseases, which might affect the overall yield. In contrast, Bohemian is more resistant but the roots are of lower value compared to those of the Common strain. The varieties can majorly be distinguished from each other by comparing the leaves, the Common variety has large crinkled leaves, while the Bohemian has smaller leaves with smooth edges. Moreover, the point of attachment of the leaf blade to the petiole is an important distinguishing mark; Common has a cordate form at the point of the leaf attachment to the petiole, whereas Bohemian has more rounded attachment angle (Courter and Rhodes, 1969).

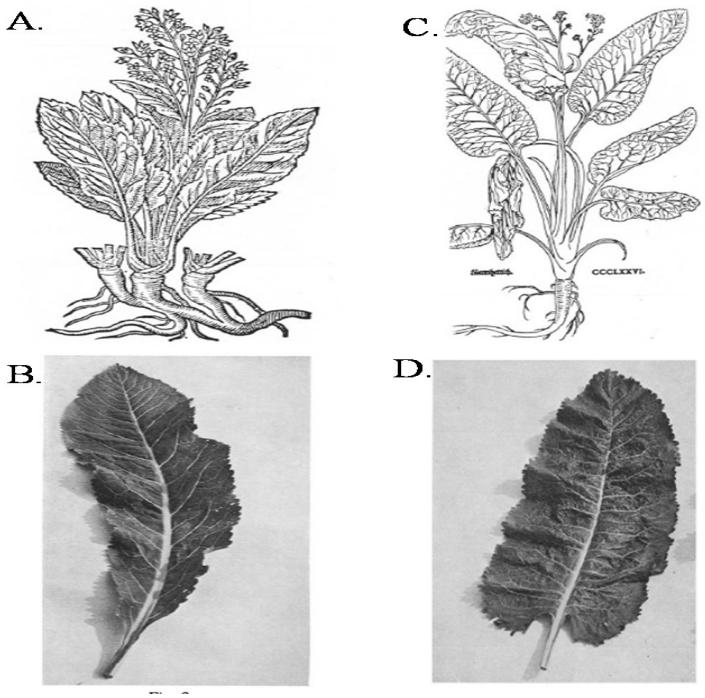


Figure 1: Sketches from Gerard´s Herbs and Fuchs´s New Kreüterbuch of the 16th century, showing different woodcuts with two leaf types (A & C). Photographs of the late 1960s showing the different leaf forms of horseradish (B & D). These sketches and photos show a striking resemblance to Bohemian variety (A & B), and Common variety (C & D) (Courtesy: Courter and Rhodes, 1969).

These characteristics show a striking resemblance to different varieties identified since the 16th century suggesting that they are preserved until our time due to the sterility and asexual propagation of this plant (Figure 1). Figure 1 shows sketches and photos of Bohemian (A &

B) and Common (C & D) horseradish plants. However, other varieties with different characteristics have also been reported (Courter and Rhodes, 1969).

2.1.3. Cultivation

Horseradish rarely produces viable seeds, and very often no seeds are found at all, therefore horseradish was frequently thought to be sterile. Due to its vegetative reproduction, horseradish reveals a low degree of variation within natural populations. Despite the reported problems in seed production, horseradish plants were generated from seeds, either by self crossing or crossing between different cultivars of horseradish. These seedlings reveal the typical heterogeneity (Weber, 1949; Courter and Rhodes, 1969).

Horseradish plants can be cultivated either as annual or perennial crop. In the annual system, the whole plants are harvested, the secondary roots are used as cuttings (sets) to grow the next generation of crops, while the large primary roots are harvested and sold as a product (Weber, 1949). When horseradish is grown as perennial crop only the major root is harvested, the secondary roots are left in the soil to grow in the following season. Productivity of perennial horseradish plantation can last until 20 years (Bratsch, 2009).

When horseradish is grown as an annual crop, sets are obtained from the secondary roots after the plant has been harvested in fall. The sets are stored in pits or cellars until they can be planted in spring. For good quality, sets should be 13 to 35 cm in length and about 0.6-1.3 cm in diameter; the main root will only increase in thickness but not in length. Sets are grown in furrows separated by about 45-60 cm, and 60 to 90 cm distance between the rows (Bratsch, 2009; Kadow and Anderson, 1940).

2.1.4. Use of horseradish

Leaves and roots of horseradish can be eaten. Yet, due to their strong pungent taste, only the fleshy roots are commercially utilized. Grated fresh roots are used as condiment with beef, when mixed with vinegar and salt, it can also be served with sea food after the addition of catsup. Many horseradish containing sauces are currently available in the market. For the US, it is estimated that 11 million kg of horseradish are annually used to produce sauces (J.R. Company, 2009).

Historically, it was the pungent flavor that raised the interest to this plant. Friese (1925) pointed out that the major pungency of horseradish is due to mustard oils. Since mustard oils are volatiles, recipes containing horseradish should not be cocked for a long time to keep their special taste (Courter and Rhodes, 1969; Friese, 1925).

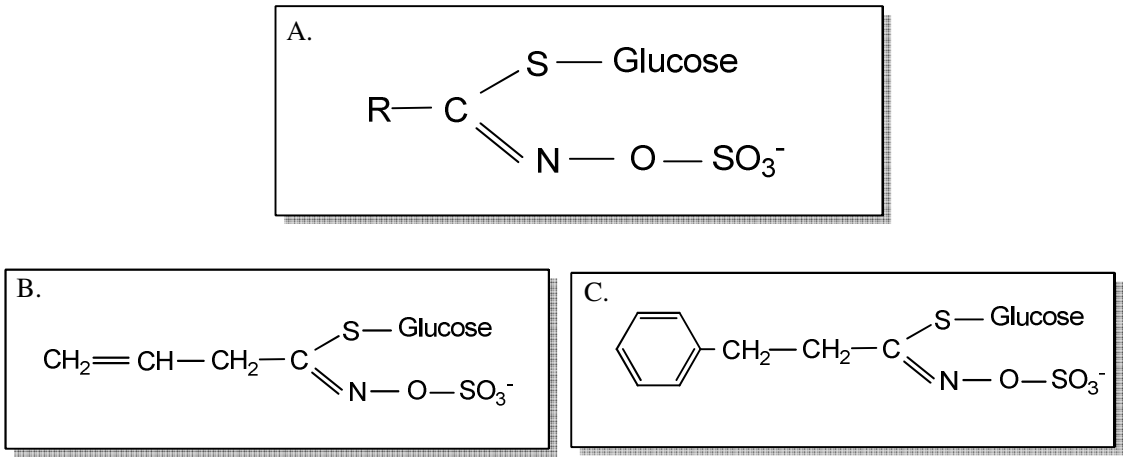


Figure 2: General structure of glucosinolates (A), sinigrin (B), and gluconasturtiin (C). R, variable side chain.

Horseradish and other *Brassica* crops contain secondary metabolite classified as glucosinolates (Figure 2, A). Upon tissue damage, glucosinolates come into contact with myrosinase (*S*-glycosidase), which degrades glucosinolates to different compounds (mustard oils), responsible for many biological effects as well as the special taste. This glucosinolate-myrosinase system is referred to as the (mustard oil bomb) (Matile, 1980).

The special taste of horseradish is majorly due to the presence of sinigrin (2-propenyl glucosinolate) and gluconasturtiin (2-phenylethyl glucosinolate), although other glucosinolates are present in minor amounts. General structure of glucosinolates and those of sinigrin and gluconasturtiin in particular are presented in Figure 2 (A-C) (Li and Kushad, 2004).

2.2. The glucosinolate-myrosinase system

2.2.1. Glucosinolates

2.2.1.1. Glucosinolate discovery, structure and distribution in the plant kingdom

Due to their presence in many important vegetables, glucosinolates (GS) have been part of human life for thousands of years (Halkier and Gershenzon, 2006). As a result of the efforts to elucidate the origin of the sharp taste of mustard seeds, the first observations on GS and their hydrolysis products, commonly known as the mustard oils, were recorded at the beginning of the 17th century. Sinigrin and sinalbin were isolated from black (*Brassica nigra*) and white (*Sinapis alba*) mustard seeds in the 1830s (Fahey et al., 2001).

The first general structure of GS was proposed in 1897 by Gadamer (Gadamer, 1897; Fahey et al., 2001). This structure proposed that the side chain is linked to the nitrogen rather than to the carbon atom in the "NCS" group. This structure was generally accepted until Ettlinger and Lundeen (1956) proposed the correct structure due to the inability of the suggested structure to explain some of these compounds properties. Moreover, in 1957 these authors described the first chemical synthesis of a GS (Ettlinger and Lundeen, 1957). Analysis of sinigrin structure by means of X-ray crystallography revealed the geometrical isomerism at the C=N bond to be Z or (anti-) (Marsh and Waser, 1970; Fahey et al., 2001).

Nowadays GS are well defined as nitrogen-sulfur containing secondary compounds derived from amino acids (Brudenell et al., 1999; Fahey et al., 2001; Johnson, 2003; Halkier and Gershenzon, 2006; Redovnikovic et al., 2008a; Hopkins et al., 2009; Selmar, 2010). There are more than 120 GS reported, all of them share a common structure (figure 2, A), consisting of a β-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester, plus a variable R group derived from one of eight amino acids. Depending on the parent amino acid, GS can be classified into aliphatic, aromatic and indolic type (Halkier and Du, 1997; Wittstock and Halkier, 2002; Canistro, 2004; Yan and Chen, 2007; Redovnikovic et al., 2008a; Selmar, 2010).

GS are found almost exclusively in the order Capparales which contains many commercially important families, such as the Brassicaceae, Capparaceae, and Caricaceae. But they are also reported from the completely unrelated genus Drypetes of the family Euphorbiaceae (Fahey et al., 2001; Mikkelsen et al., 2002; Wittstock and Halkier, 2002; Halkier and Gershenzon,

2006). A wide variation of GS distribution is noticed between different families and also organs of GS producing plants (Mikkelsen et al., 2002; Brown et al., 2003; Hopkins et al., 2009).

In near history, initial interest in GS was to enhance the quality of rapeseed meal by reducing the GS content of rapeseed, because oxazolidine-2-thione is formed upon hydrolysis of 2-hydroxy-3-butenyl GS, one of the predominant GS in rapeseed, which causes goiter and other harmful effects to animal fed on rapeseed meal (Haughn et al., 1991; Halkier and Gershenzon, 2006). Therefore, plant breeders focused on producing commercial rapeseed varieties (double low), which contain low levels of GS and erucic acid. Unfortunately, this was achieved with high cost regarding crop protection (Fahey et al., 2001; Hopkins et al., 2009).

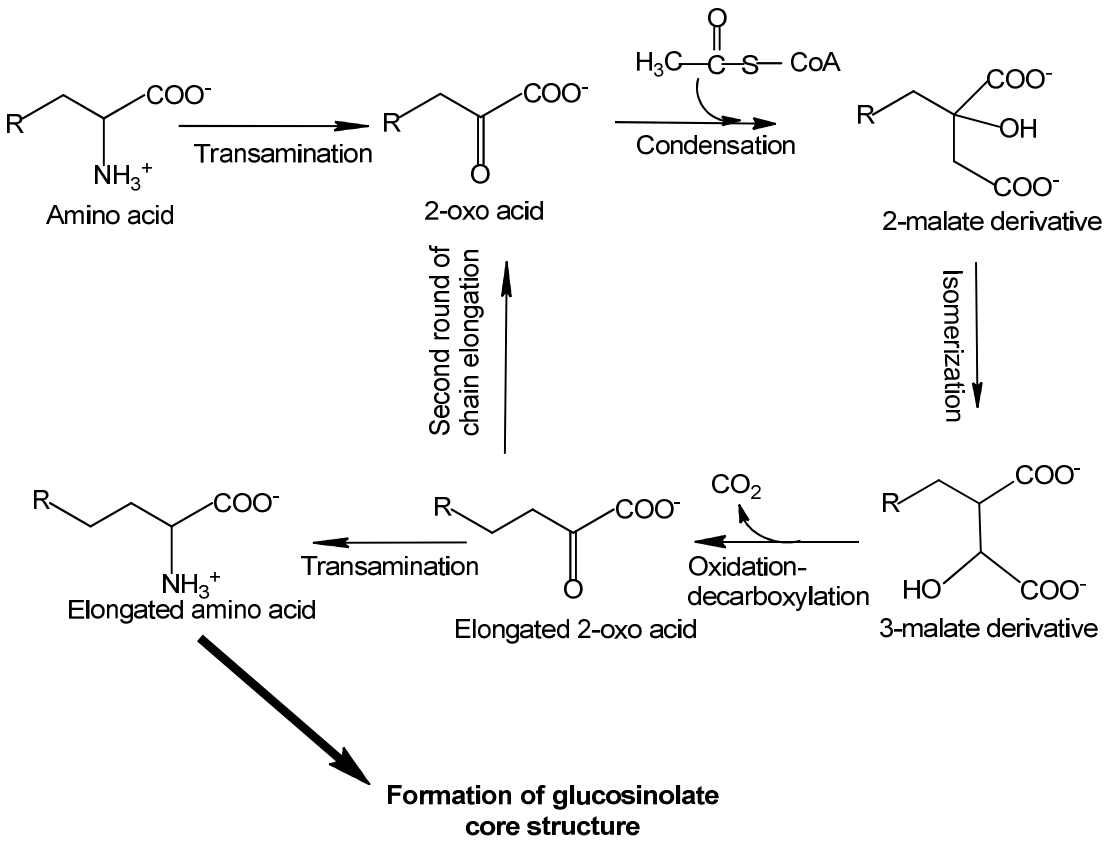


Figure 3: Amino acid chain elongation in the glucosinolate biosynthesis pathway. Abbreviations: R, variable side chain.

2.2.1.2. Glucosinolate biosynthesis

GS biosynthesis has been thoroughly reviewed in recent years (Halkier and Du, 1997; Mikkelsen et al., 2002; Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006; Redovnikovic et al., 2008a; Selmar, 2010). The GS biosynthesis can be divided into three steps: Amino acid chain elongation, core structure biosynthesis and secondary modifications.

2.2.1.2.1. Amino acid chain elongation

Many GS are not directly derived from protein amino acids, but from corresponding derivates. These are synthesized by chain elongation of the parent amino acid (Figure 3). Firstly, 2-oxo acid is produced through transamination, followed by a condensation reaction with acetyl-CoA, and isomerization of the 2-malate derivative to the 3-malate derivative. Then one of the two carbon atoms is lost through decarboxylation to result in an elongated 2-oxo acid. The elongated 2-oxo acid can either be transaminated to produce the elongated amino acid and continue to the second step of GS biosynthesis. Alternatively, it can undergo other cycles of chain elongation, up to nine cycles might occur in plants (Fahey et al., 2001; Halkier and Gershenzon, 2006; Selmar, 2010).

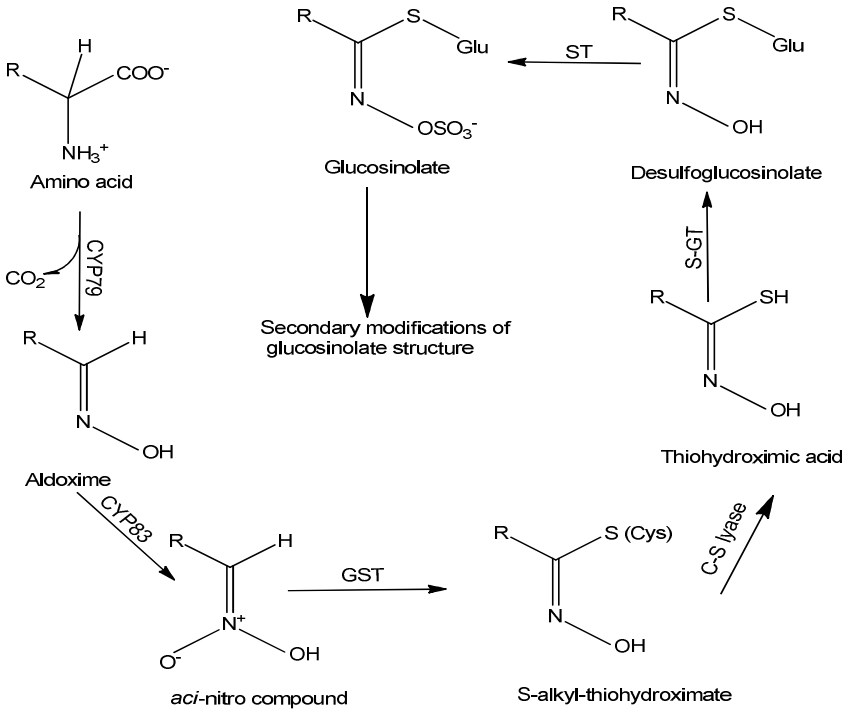


Figure 4: Biosynthesis of glucosinolate core structure. Abbreviations: R, variable side chain; GST, glutathione-S-transferase; S-GT, S-glucosyltransferase; ST, sulfotransferase; Glu, glucose.

2.2.1.2.2. Biosynthesis of glucosinolate core structure

The biosynthesis of GS core structure is common to all GS (Halkier and Gershenzon, 2006), and meanwhile most of the intermediates of this pathway are known (Wittstock and Halkier, 2002). The occurrence of GS in *Arabidopsis* facilitates the identification and characterization of genes encoding the key enzymes of the GS biosynthetic pathway. (Halkier and Gershenzon, 2006). Figure 4 shows the different steps of amino acid core structure formation.

2.2.1.2.2.1. Aldoxime formation

The first step in GS core structure formation is catalyzed by cytochrome P450, which belongs to the CYP79 family; P450 converts the precursor amino acids to aldoximes (Wittstock and Halkier, 2002). Functional genomic approaches were used to identify most of the seven CYP79s based on the similarity of GS and cyanogenic glucocide biosynthetic pathways, which both share aldoximes as intermediates (Halkier and Du, 1997; Mikkelsen et al., 2002; Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006). Different approaches were used to identify the functions of different CYP79s; CYP79 homologs were heterologously expressed and characterized with respect to their substrate specificity after being identified in *A. thaliana*. Yeast screens for cDNAs conferring resistance to 5-fluroindole led to CYP79B2 isolation, which along with its homologous CYP79B3 is responsible of tryptophan conversion to indole-3-acetaldoxime (IOAx). A double knockout of these genes results in removal of indole glucosinolates, showing that no other genes significantly contribute to indole glucosinolate biosynthesis (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006; Hull et al., 2000; Mikkelsen et al., 2000; Zhao et al., 2002).

The function of CYP79F1 and its homologous CYP79F2 was revealed in independent genetic approaches, where CYP79F1 mutants *bushy* and *supershoot* lack short chain aliphatic glucosinolates (Tantikanjana et al., 2001; Reintanz et al., 2001). From these findings it was deduced that CYP79F1 produces short-chain methionine derivatives (up to four additional methylene groups), while CYP79F2 is responsible for the production of longer chain methionine derivatives (Reintanz et al., 2001). However, biochemical studies showed that CYP79F1 produces both short and long chain methionine derivatives (one to six additional methylene groups), while CYP79F2 is responsible for the production of the longer ones (Hansen et al., 2001b; Chen et al., 2003). From these results it is important to characterize

proteins encoded by different genomes, since assumptions based on genetic studies might be misleading (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006).

CYP79A2 is responsible for the biosynthesis of GS derived from phenylalanine (Wittstock and Halkier, 2002). The function of the remaining CYP79C1 and CYP79C2 is unknown, but due to their low transcript levels, it is suggested that they might be responsible for the biosynthesis of aldoxime of infrequently occurring GS, which are often derived from methionine, homophenylalanine, and tyrosine (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006).

2.2.1.2.2.2. Thiohydroximic acid formation

Two cytochromes have been found to mediate the metabolism of aldoximes. The first, CYP83B1, was identified using biochemical and genetic approaches (Bak and Feyereisen, 2001; Barlier et al., 2000, Delarue et al., 1998; Hansen et al., 2001a; Smolen and Bender., 2002). The second gene CYP83A1 is a homolog of CYP83B1 but metabolizes majorly aliphatic aldoximes (Naur et al., 2003; Bak and Feyereisen., 2001). Both enzymes metabolize aromatic aldoximes, with CYP83B1 showing higher affinity to aromatic aldoximes than CYP83A1. Both enzymes produce an *aci*-nitro or nitrile oxide, which could not be isolated *in vivo* due to their instability. However, the product reacts *in vitro* with nucleophilic S-donor to form S-alkyl thiohydroximates (Bak and Feyereisen., 2001; Hansen et al., 2001a). It is proposed that a glutathione S-transferase like enzyme might control cysteine (the proposed S donor) interaction with the intermediate, which ensures the conjugation with the S-donor *in vivo*. (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006). 2-substituted thiazoline-4-carboxylic acid is produced *in vitro* by internal cyclization of S-(hydroximoyl)-L-cysteine conjugates. This can be explained by tight coupling of CYP83 enzymes to the sulfur donating enzymes, which are coupled to C-S lyase, the enzyme responsible of S-alkyl thiohydroximate cleavage to thiohydroximic acid (Halkier and Gershenzon, 2006). C-S lyase seems to be coded by a single gene family, since knockout mutants of C-S lyase showed complete removal of aliphatic and aromatic glucosinolates (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006).

2.2.1.2.2.3. Glucosinolate formation

The final step of GS core structure formation is catalyzed by UDP-glucose:thio-hydroximic acid S-glucosyltransferase (UGT). Corresponding desulfoglucosinolates are produced by UGT74B1 through glycosylation of phenylacetothiohydroximic acid. The presence of additional UGTs in the genome is proposed, since knockout of this enzyme did not abolish GS accumulation completely. Different PAPS:desulfoglucosinolate sulfotransferases were found to catalyze the last step of core structure synthesis, and they show to prefer different precursors; long chain aliphatic desulfoglucosinolates are preferred by AtST5b and AtST5c, while AtST5a prefers tryptophan and phenylalanine-derived desulfoglucosinolates (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006; Piotrowski et al., 2004).

2.2.1.2.3. Secondary modifications

After the synthesis of the primary or parent GS, it may undergo a variety of modifications of the side chain and glucose group. These modifications are of great importance since they affect the hydrolysis products, and increase the number of different GS enormously. The major modifications include: Oxidation, hydroxylation, methoxylation, desaturation, sulfation and glycosylation. These modifications are known to have -organ and development- specific pattern (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006; Piotrowski et al., 2004; Redovnikovic et al., 2008a).

2.2.1.3. Regulation of biosynthesis

GS biosynthesis is genetically as well as environmentally regulated. Some GS are constitutively present, while others can be induced (Mikkelsen et al., 2002; Halkier and Gershenzon, 2006). Different plant cultivars have different concentrations of GS. Sulfur and nitrogen supply affect GS levels, other exogenous factors such as drought and NaCl also affect GS concentrations (Falk et al., 2007; Selmar and Kleinwächter, 2013; Li and Kushad, 2004; Jensen et al., 1996; Keling and Zhujun, 2010).

Different signal compounds have different effects on GS accumulation, for example, methyl jasmonate (MeJA) induces some indole glucosinolates. Salicylic acid treatment of *Brassica napus* increases 2-phenylethyl glucosinolate (gluconasturtiin) accumulation. Aliphatic GS in *Arabidopsis* appear to be developmentally regulated, although some exceptions were reported

(Bodnaryk, 1992; Bodnaryk, 1994; Brader et al., 2001; Mikkelsen et al., 2003; Kliebenstein et al., 2002; Kiddle et al., 1994).

2.2.1.4. Glucosinolate transport in plants

Different plant organs accumulate different amounts of GS. In *Arabidops*is high concentrations were found in young leaves and reproductive tissues. Intermediate concentrations were found in roots, leaves and stems. Senescing leaves contain the lowest GS concentrations (Brown et al., 2003).

GS transport to seeds was suggested, since their accumulation in seeds is not accompanied by a high biosynthetic activity (Nour-Eldin and Halkier, 2009; Du and Halkier, 1998). This suggestion is strengthened by several observations; GS have physiochemical properties necessary for long distance transport. GS concentrations up to 10 mM were found in phloem by analyzing aphids feeding phloem sap (Halkier and Gershenzon, 2006; Merritt, 1996). Feeding studies of *Arabidopsis* leaves showed that ^{14}C labeled GS were found in seeds following translocation through the phloem (Chen et al, 2001). *Brassica napus* protoplast and embryo specific uptake of a variety of GS against a concentration gradient suggests the presence of receptors that might play a role in GS transport (Chen and Halkier, 2000; Gijzen et al., 1989).

Yet, until now the transport form of GS needs to be clarified. Indeed, there are evidences supporting the transport of intact GS or desulfoglucosinolates (Nour-Eldin and Halkier, 2009; Brundell et al., 1999). However, the apoplastic occurrence of myrosinase, which degrades GS, points out, that any apoplastic passage, e.g., during phloem loading, must be performed via a hydrolysis-resistant transport form, such as diglucosidic metabolites.

2.2.1.5. Glucosinolate degradation

Upon tissue disruption, for example, by mechanical damage or insect injury, GS and myrosinase come into contact. As consequence, GS are hydrolyzed, resulting in a glucose molecule and an unstable aglycone. The latter one rearranges depending on various factors to numerous products, such as isothiocynates, nitriles and thiocynates. The corresponding mixtures of these hydrolysis products are known as mustard oils (Figure 5).

A lossen rearrangement of the aglycone moiety results in isothiocyanate formation. The isothiocyanates formed from GS revealing hydroxyl groups on C-2 of their side chain are unstable and cycle to oxazolidine-2-thiones, which cause goiter. Epithiospecifier proteins (ESP) are involved in nitrile or epithionitrile *in vivo* formation depending on the side chain structure (Bernardi et al., 2000; Foo et al., 2000; MacLeod and Rossiter, 1985; Tookey, 1973; Lmbrix et al., 2001). *In vitro* formation of nitriles occurs in media at low pH or in the presence of Fe^{2+}. In the presence of thiocyanate forming proteins (TFP), thiocyanates mainly are formed from benzyl-, allyl- and 4-methylsulfinylbutyl-glucosinolate. Unlike hydrolysis from other GS types, isothiocyanates generated from indolic GS are unstable and convert to other compounds, such as indole-methanols, which subsequently undergo various further reactions producing ascorbic acid conjugates, or oligomeric mixtures (Latxague et al., 1991; Buskov et al., 2000; Agerbirk et al., 1998; Gil and MacLeod, 1980; Halkier and Gershenzon, 2006; Hasapis and MacLeod, 1982).

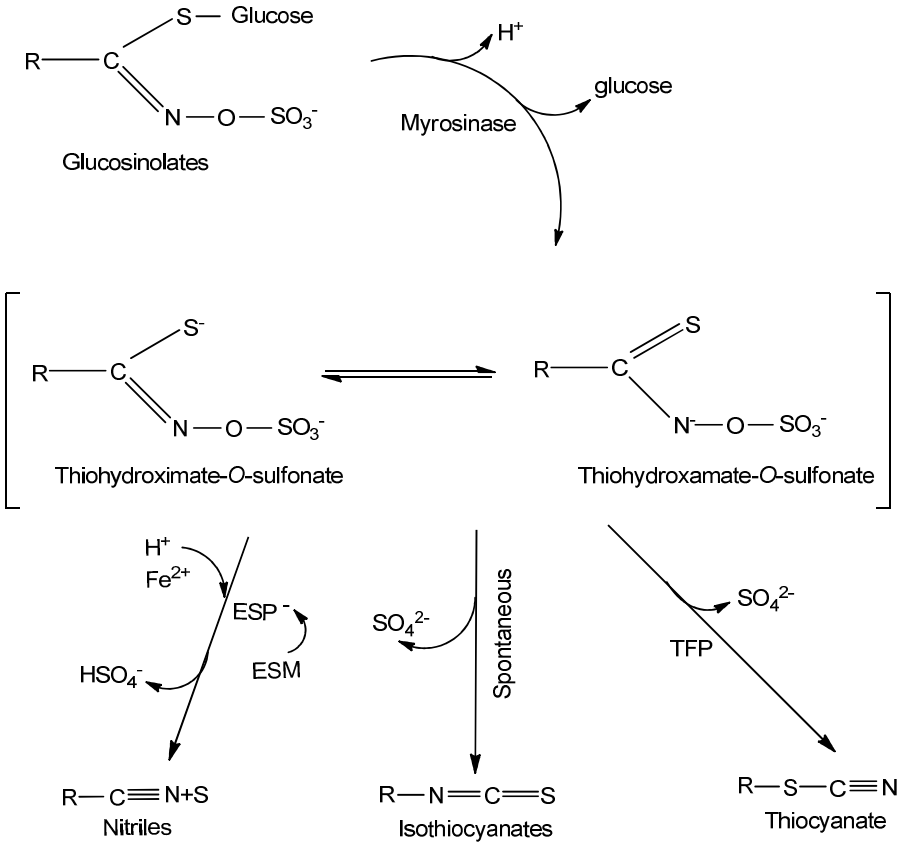


Figure 5: GS hydrolysis by myrosinase and mustard oils formation. Abbreviations: R, variable side chain; TFP, thiocyanate forming proteins; ESP, epithiospecifier proteins; ESM, epithiospecifier modifier.

2.2.2. Myrosinase

Myrosinases (E.C.3.2.3.1), represent ß-thioglucosidase enzymes, which catalyze GS hydrolysis (Brown et al., 2003; Bones and Rossiter, 1996). Myrosinases were discovered in mustard seeds by Bussy in 1840. All plant species containing GS also contain myrosinases. Moreover, myrosinases were reported from bacteria, fungi, and insects (Bones and Rossiter, 1996). It was thought that myrosinase in aphids might be obtained from their host plant. However, these myrosinases did not cross react with antibodies raised against plant myrosinases, which indicates that these enzymes are not derived from the plant host but must be synthesized by the insect (Bones and Rossiter, 1996).

The three dimensional structure of myrosinase shows that the enzyme has abundant salt bridges, disulfide bridges, and H bonds, which might help to maintain enzyme stability in extracellular environments. Moreover, more stability and protection against hydrolysis products might be due to the heavy glycosylation of the enzyme, where carbohydrate might compose up to 20% of the total molecular mass (Halkier and Gershenzon, 2006); fucose, mannose and *N*-acetylglucosamine are the main carbohydrates (Rask et al., 2000).

Myrosinases act only on GS as their sole substrate and reveal no activity toward *O*-glycosides or other naturally occurring *S*-glycosides (Halkier and Gershenzen, 2006). However, their specific activity among different GS may differ. Some myrosinases are known, which hydrolyze various substrates, but others reveal high substrate specificity (Bernardi et al., 2003; MacLeod and Rossiter, 1986). Myrosinases are encoded by multigene familiy ranging from 4 in the model plant *Arabidopsis* to 20 or more genes in *Brassica napus* and *Sinapis alba.* These genes show organ and tissue specific expression (Halkier and Gershenzon, 2006).

2.2.2.1. Myrosinase gene structure and isozymes

Genetic studies of different myrosinases genes revealed sequence similarity and similar intron-exon arrangement. All functional myrosinase genes studied were conserved in 12 exons separated by 11 introns (Lenman et al., 1993b; Thangstad et al., 1993; Xue et al., 1995). Interestingly, upon comparison of *Arabidopsis* myrosinase genes with those of *O-ß*-glucosidases from monocotyledons and dicotyledons origin, similar genetic organization of introns and exons was noticed, although some differences were found. This similarity of intron-exon organization between myrosinases and *O-ß*-glucosidases provides strong

evidence that both enzymes have a long common evolutionary history, although the structure of their ancestral genes still needs to be elucidated (Rask et al., 2000).

cDNA isolation and genomic clones led to the identification of several subfamilies of myrosinase (Chadchawan et al., 1993; Falk et al., 1992; Falk et al., 1995a; Lenman et al., 1993a; Machlin et al., 1993; Thangstad et al., 1993; Xue et al., 1992; Xue et al., 1995, Xue and Rask, 1995). Three subfamilies were found in studies with Brassicaceae, they were designated; MA (myr 1), MB (myr 2) and MC.

The number of myrosinase isoforms and that of the genes encoding them differs among individual species (Rask et al., 2000). In *Arabidopsis thaliana* three different genes encoding myrosinase were found (Xue et al., 1995, Xue and Rask, 1995). In *Brassica napus* seeds myrosinase subunits with different sizes were found, ranging from 75 kDa for MA, to 65 kDa for MB (Lenman et al., 1993a; Falk et al., 1995a). These size differences can be explained by different glycosilation degrees. Interestingly, under non-denaturing conditions only MA was isolated as free dimer. In addition, MB and MC were complexed with other proteins (Lenman et al., 1990).

The expression of different myrosinase isoforms was studied in *Brassica napus* and *Sinapis alba*, in both species MA genes were expressed exclusively in the myrosin cells of the seed embryo, while those of MB were found in the cotyledons. In *Brassica napus* the expression of MC genes was also detected in the seed embryo. Investigations of the expression of different myrosinase subfamilies in different organs and developmental stages showed the occurrence of myrosinases with different sizes in different developmental stages (Falk et al., 1992; Falk et al., 1995a; Lenman et al., 1993a; Xue et al., 1995; Rask et al., 2000).

2.2.2.2. Myrosinase reaction and role of ascorbic acid

The three dimensional structure of *Sinapis alba* myrosinase has been revealed since 1997, it shows $(\alpha/\beta)_8$ barrel structure, which is also common to other glucosidases. The general structure of myrosinase is similar to that of linamarase, although both enzymes share only 46% sequence identity. Myrosinases are homodimers that contain three disulfide bridges, which stabilize the N-terminus of the enzyme. Furthermore, a zinc atom surrounded by two amino acids located at the myrosinase interface coordinate the enzyme dimers (Rask et al., 2000; Burmeister et al., 1997).

Myrosinase reaction proceeds in two steps; firstly, a glutamic acid residue acts as a nucleophil attacking the glucosinolate substrate, forming a covalent intermediate with concomitant aglycone departure. Secondly, a water molecule attacks the enzyme-aglycone intermediate causing the liberation of the glucose residue (Burmeister et al., 1997; Rask et al., 2000).

Myrosinases can usually metabolize a wide range of GS with different side chain structures. It seems that a hydrophobic pocket with conserved amino acid sequence is responsible for binding the hydrophobic part of the aglycone moiety. This pocket is located near the active site at the enzyme C terminus (Rask et al., 2000)

Ascorbic acid has been reported to co-localize with GS in the cell vacuole (Bones and Rossiter, 1996). Its importance in the glucosinolate-myrosinase system rose from its considerable activating effect on myrosinase (Ettlinger et al., 1961; James and Rossiter, 1991; Kleinwächter and Selmar, 2004). More than 99% of horseradish roots ascorbic acid is located in the cell vacuole (Bones and Rossiter, 1996). Mean ascorbic acid concentration in root tissue is 2 mM, indicating that the concentration must be much higher in vacuoles, where it is mainly stored (Bones and Rossiter, 1996).

Myrosinases have one site for binding GS with two moieties, one for the glycone and the other for the aglycone part, and another binding site for its activator ascorbic acid (Bones and Rossiter, 1996). Ascorbic acid might also interact with the active centre of the enzyme. Upon ascorbic acid binding to the enzyme, myrosinase undergoes conformational changes, leading to increased enzyme activity. However, when ascorbic acid concentration is too high, it might interfere with the active centre leading to enzyme inhibition (Bones and Rossiter, 1996; Ohtsuruo and Hata, 1979).

Although the role of ascorbic acid as activator of myrosinase has been recognized since a long time, most of the related studies did not consider adequately that almost all myrosinase activity determination methods are interfered by ascorbic acid. Kleinwächter and Selmar (2004) reviewed several methods used to measure myrosinase ractivity and pointed out the limitations of these methods. Moreover, these authors developed an ascorbic acid insensitive method to determine myrosinase activity (Kleinwächter and Selmar, 2004).

2.2.2.3. Myrosinase related proteins

2.2.2.3.1. Myrosinase binding proteins

Myrosinase binding proteins (MBP) were first described in 1990, when several polypeptides co-precipitated with anti-myrosinase antibodies (Bones and Rossiter, 1996; Lenman et al, 1990). These proteins consisting of repeated residues are thought to be formed by alternative splicing of their corresponding mRNA or proteolytic cleavage of their protein precursors (Rask et al., 2000).

Their actual function is unknown. However, it was found that the synthesis of these proteins is induced by wounding and methyl jasmonate treatment (Geshi and Brandt, 1998; Taipalensuu et al., 1997b). MBP are similar to lectins, and this suggests that MBP might synergize the effects of the GS hydrolysis products by binding directly to carbohydrates of insects or fungi (Rask et al., 2000; Kissen et al., 2009).

Alternatively, it was discussed that MBP might stabilize myrosinase structure. This hypothesis was confirmed by the finding that MBP antisense *Brassica napus* plants failed to produce the regular myrosinase complex (Kissen et al., 2009; Eriksson et al., 2002). A study on *Brassica napus* cotyledons revealed that myrosinase and MBP are colocalized at the cellular and sub-cellular levels. This suggests that myrosinase forms large complex structures in plants. However, at other stages of plant development myrosinase and MBP were observed in different cells. This suggests that myrosianse complex is formed only after tissue damage (Eriksson et al., 2002; Andreasson et al., 2001; Kissen et al., 2009; Geshi and Brandt, 1998).

In addition, further proteins of *Brassica napus* extracts reacted with monoclonal antibodies against MBP. However, since these proteins were not able to complex with myrosinase, they were designated as myrosinase binding protein related proteins (MBPRP). Functions of MBPRP are so far unknown (Bones and Rossiter, 1996).

2.2.2.3.2. Myrosinase associated proteins

Myrosinase associated proteins (MyAP) are another class of proteins found to complex with MB and MC myrosinases in *Brassica napus* seeds (Taipalensuu et al., 1996). These 40 kDa monomeric glycoproteins show sequence similarities with *Arabidposis* lipase, which suggests that MyAP function is to facilitate acylated GS hydrolysis by releasing the acyl group from

acylated GS. In *A. thaliana* a MyAP was found to interact with epithiospecifier proteins (ESP; see below). These proteins, referred to as (epithiospecifier modifier 1 (ESM1)), and show to affect GS degradation products (Zhang et al., 2006; Rask et al., 2000; Kissen et al., 2009)

Methyl jasmonate and wounding induced the synthesis of MyAP isoforms in vegetative tissues, while no effect was noticed on those MyAP expressed in the seeds of *Brassica napus*. Moreover, MyAP are expressed differently in various organs and developmental stages of *Brassica napus*. Until now, it is not known if the differentially expressed MyAP have the same or different functions (Rask et al., 2000; Kissen et al., 2009; Taipalensuu et al., 1997a).

The location of MyAP stills a matter of debate. Studies investigating MyAP location could not elucidate the cell type, where the constitutive MyAP were transcribed. Moreover, upon induction, MyAP transcripts were identified in nearly all cells types. In conclusion, MyAP are suggested to be located in vacuoles, peroxisomes and endoplasmic reticulum (ER) at the sub-cellular level (Rask et al., 2000; Kissen et al., 2009; Andreasson et al., 1999).

2.2.2.3.3. Epithiospecifier proteins

Epithiospecifier proteins (ESP) were first isolated from *Crambe abyssinica* seeds (Tookey, 1973), and not all Brassicaceae species and even ecotypes of the same species seem to possess ESP (Kissen et al., 2009).

ESP don´t cause GS degradation, but in the presence of ferrous ions they shift myrosinase degradation of GS toward nitriles and epithionitriles formation. Yet, the underlying mechanism of this reaction shift is still unknown. However, in *Brassica napus* ESP have no activity in the absence of ferrous ions. A unique character of ESP is that they solely specify the reaction products and not the substrate (Bones and Rossiter, 1996; Kissen et al., 2009).

ESP were shown to be expressed in almost all organs of *A. thaliana*. Differential ESP expression was also observed in different cycles of plant growth. Data, although not conclusive, suggests that ESP are localized in cells adjacent to cells expressing myrosinase in *A. thaliana*. It is assumed that ESP and GS localization might overlap in some tissues. (Kissen et al., 2009; Burow et al., 2007). Independent studies showed that ESP are located in the cytoplasm and nucleus at the sub-cellular level, which is different from all other so far

identified locations of other components of the myrosinase-glucosinolate system (Kissen et al., 2009; Burow et al., 2007).

2.2.3. Evolution of the glucosinolate-myrosinase system

2.2.3.1. Evolution of glucosinolate

GS seem to be developed from the evolutionary older and taxonomically wider distributed cyanogenic glycosides, several lines of evidence support this assumption; both groups are produced from amino acids, and all five amino acids used to produce cyanogenic glycosides are also used for GS biosynthesis. Moreover, both groups share similar enzymes and intermediate in their biosynthesis pathway (Halkier and Gershenzon, 2006; Selmar, 2010).

2.2.3.2. Myrosinase evolution

On the basis of amino acid similarities, *O*-glycosyl hydrolases or glycosidases (EC 3.2.1-) superfamily was divided into several families. Databases are continuously updated, and 70 families have been already reported. Despite of their recognition as thioglucosidases, myrosinases were included in family 1 (clan GH-A) of *O*-glucosidases along with enzymes from prokaryotic and eukaryotic origin (Rask et al., 2000).

Myrosinases from *Sinapis alba* seeds and linamarase (cyanogenic ß-glucosidase) are quite similar except of their specificity for the *S*- or *O*-glycosidase. Both enzymes are glycosylated homodimers, similar in size, have overall general structure and accept various substrates. Moreover, both enzymes share $(ß/α)_8$ barrel structure and a hydrophobic pocket in the C-terminal substrate binding site (Burmeister et al., 1997; Rask et al., 2000).

2.2.4. Localization of the glucosinolate-myrosinase system in plants

Guignard was the first to use the term “myrosin” to describe a special type of cells discovered in Brassicaceae by Heinricher in 1884 (Bones and Rossiter, 1996; Rask et al., 2000). Myrosin cells, once referred to as protein accumulating idioblasts and myrosin tubes are different from other cells in terms of size and morphology (Rask et al., 2000). The presence of myrosinase in the myrosin cells, which are known to occur in different plant organs, was confirmed using different methods such as histochemical techniques, immunological studies using antibodies against myrosinase and *in situ* hybridization experiments (Thangstad et al., 1990; Höglund et al., 1991; Falk et al., 1995a; Lenman et al., 1993a; Xue et al., 1995). Apart from its

localization in myrosin cells, myrosinase also has been shown to occur in guard cells (Rask et al., 2000).

Also with respect to the subcellular localization of myrosinases, in part, contradictory findings have been reported. Lüthy and Matile (1984) proposed the mustard oil bomb hypothesis, which suggested that GS are localized in the vacuole, whereas myrosinase is accumulated in myrosin grains or associated with the plasma membrane of the same cells. However, antibodies generated against myrosinases and sinigrin showed that myrosinases are localized in myrosin cell, while GS are stored in the vacuole of non myrosin cells, indicating that myrosinase and GS are localized in different cell types (Rask et al., 2000; Höglund et al., 1992; Kelly et al., 1998; Thangstad et al., 1991). These observations were complemented by the finding that GS in *Arabidopsis* flower stalks are localized in specific elongated, sulfur rich cells (S cells) (Koroleva et al., 2000). Thus, a cellular compartmentation of the single components of the glucosinolate-myrosinase system seems to be more likely than subcellular one.

In contrast, both GS and myrosinase were found to be co-localized in aleurone-type cells in *Brassica juncea* seeds and seedlings (Kelly et al., 1998). This points out that GS and myrosinase might also be separated at the sub-cellular level or localized in the same compartment; in this case myrosinase might be deactivated by high ascorbate concentrations (Halkier and Gershenzon, 2006; Bones and Rossiter, 1996).

2.3. Significance of glucosinolates

2.3.1. Ecological role

2.3.1.1. Plant interactions with insects and herbivores

The hydrolysis products of GS are described to act as deterrent and poison against generalist insects and herbivores, such as birds and slugs. Albeit, some insects evolved mechanisms to tolerate the negative effects of GS hydrolysis products. Moreover, they used GS and their hydrolysis products as cues for feeding and egg lying (Martin and Müller, 2007; Hopkins et al., 2009; Textor and Gershenzon, 2009; Redovnikovic et al., 2008a).

Plants accumulating GS generally reveal a high degree of resistance against herbivores. Herbivore feeding was reported to elicit an increase of GS contents in the tissue. The highest enhancements usually are found for indole GS (Martin and Müller, 2007; Redovnikovic et al., 2008a; Textor and Gershenzon, 2009). However, exceptions have also been reported; in *A. thaliana* aliphatic GS increased, upon infestation with specialist and generalist aphids as well with a generalist caterpillar, whereas indole GS showed no significant difference (Mewis et al., 2005).

GS and their hydrolysis products are known to attract specialist insects. The volatile hydrolysis products might work as attraction signal of these insects, but they also might attract the natural enemies of these insects, which help to protect the plants (Mewis et al., 2002; Miles et al., 2005; Rojas, 1999; Mattiacci et al., 1994).

Various mechanisms are realized by specialist herbivores to overcome the toxicity of GS and their hydrolysis products; Wittstock et al. (2004) reported that *Pieris rape* accumulates nitrile-specifier proteins in its gut to direct GS hydrolysis from isothiocyanates towards the production of the less toxic nitriles, which are excreted later with the feces (Wittstock et al., 2004). Many specialists are known to sequester GS in their tissues and exploit them for self-protection, in order to do so, they either use their own myrosinase or depend on myrosinases found in the gut of their enemies (Aliabadi et al., 2002; Müller et al., 2001; Bridges et al., 2002; Jones et al., 2002). Upon insect damage, GS hydrolysis products produced may also act as a signal to alarm other members of the colony (Bridges et al., 2002). *Plutella xylostella* disarms the glucosinolate-myrosinase system by using its own sulfatase enzyme, the resulting desulfoglucosinolate is no longer hydrolyzed by myrosinase. Moreover, all GS major groups

can be modified by *P. xylostella,* which enables it to grow on different Brassicaceae plants (Ratzka et al., 2002).

2.3.1.2. Plant interaction with microorganisms

Whereas many studies have demonstrated the protective role of GS hydrolysis products against herbivores, fewer studies have been conducted concerning plant pathogen resistance (Redovnikovic et al., 2008a; Smolinska et al., 2003; Mari et al., 2002; Li et al., 1999).

Fungi have been reported to be more susceptible to aromatic isothiocyanates than to aliphatic ones. The analysis of *Arabidopsis* crude extracts showed that 4-methylsulphynilbutyl isothiocyanate inhibits different pathogens; *Pseudomonas syringae* showed 50% growth inhibition *in vit*ro. Results of further experiments with wild type and mutant plants susceptibile to different bacteria and fungi, led to the suggestion that GS hydrolysis products confer resistance against particular pathogens (Tierens et al., 2001; Redovnikovic et al., 2008a).

After inoculation with *Sclerotinia sclerotiorum*, different *Brassica napus* cultivars responded differently. They showed local and systemical changes in GS patterns, and the total concentrations were increased (Redovnikovic et al., 2008a; Li et al., 1999). More recently different *Arabidopsis thaliana* mutants with reduced indolic and aliphatic GS contents were shown to be very sensitive to *S. sclerotiorum.* Moreover, mutant plants (myb28), which biosynthesize reduced levels of C3, C4 and C6-C8 aliphatic GS, were more susceptible to *S. sclerotiorum* than wild type plants. In corresponding *in vitro* studies, long side chain isothiocyanates turned out to reveal the highest toxic activity against *S. sclerotiorum* (Stotz et al., 2011).

2.3.2. Agricultural relevance

2.3.2.1. Break crops and biofumigation

GS and their hydrolysis products also show inhibitory effects against soil-borne pathogens. *Gaeumannomyces graminis* (Ggt) is a fungus causing diseases in wheat. Its growth was inhibited *in vitro* after treatment with isothiocyanate amounts, which resemble the amounts released upon *Brassica* crops degradation in soil. These findings increased the interest of the use of *Brassica* species as soil biofumigant as alternative to chemical ones (Angus et al.,

1994; Sarwar et al., 1998). This has led to the recognition of *Brassica* crops as so called break crops. Break crops are used to break the life cycle of soil-borne organisms. It turned out that wheat grown after *B.napus* cultivation revealed yield increase by 19% when compared with two rotations of wheat crops. This increase seems to be associated with less pathogen pressure, than being an effect of residual nutrients or water. Disease control seems to be a synergistic effect of Ggt host absence and Ggt growth inhibition (Angus et al., 1994; Angus et al., 2011; Sarwar et al., 1998).

2.3.2.2. Antinutritional effects of glucosinolates in animal feed

B. napus (rapeseed), containing about 40% oil in the seeds is one of the most important oil producing crops in the world. The rapeseed meal (the cake left after pressing the seeds to extract oil) is considered as an optimal animal feed due to its high content of proteins. Unfortunately, the presence of GS reduced the nutritional value of rapeseed meal, in 1970s plant breeding programs were designed to produce rapeseed cultivars with low GS (Fahey et al., 2001; Cartea and Velasco, 2008).

The presence of GS in animal feed reduces its palatability, and affect animal growth, performance as well as reproduction. Moreover, GS cause goiters and internal organ abnormalities (Tripathi and Mishra, 2007; Mawson et al., 1995). Upon progoitrin hydrolysis by myrosinase, oxzalidine-2-thione is released. The later compound is known for its goitrogenic effect on mammals by inhibiting iodine uptake in the thyroid, which causes an enlargement of the thyroid (Bones and Rossiter, 1996; Fahey et al., 2001). Moreover, other GS hydrolysis products, such as nitriles, affect kidney and liver functions (Tripathi and Mishra, 2007; Verkerk et al., 2009).

Mawson et al. (1993) indicated that younger animals, such as chicks, piglets and calves, are more sensitive to the negative effect of GS hydrolysis products, and that feed palatability can be increased by using rapeseed meals with reduced GS levels (Mawson et al., 1993).

2.3.3. Glucosinolate and plants development and growth

2.3.3.1. Plant growth regulation

Indole GS are assumed to affect plant development, since these compounds correspond to precursors of the plants hormone indole-3-acetic acid (IAA). Several lines of evidence

support such assumption; upon indole GS hydrolysis, indole acetonitrile (IAN) is released, which can further be hydrolyzed by nitrilase to form IAA (Searle et al., 1982; Bartel and Fink., 1994). In the indole GS biosynthesis pathway, the first formed intermediate is indole-3-acetaldoxime, which is now considered a branch point between the metabolic pathways resulting in indole GS or IAA formation (Bak et al., 2001). Studies with *Arabidopsis* mutants not only showed the role of indole-3-acetaldoxime as a branch point for either IAA or indole GS synthesis, but also suggested the presence of another pathway for IAA synthesis. Moreover, indole-3-acetaldoxime was found to be a precursor for camalexin (Redovnikovic et al., 2008a; Bak et al., 2001, Mikkelsen et al., 2004; Zhao et al., 2002; Bender and Celenza, 2008).

2.3.3.2. Impact of sulfur and nitrogen on glucosinolate accumulation

GS contain sulfur and nitrogen. Accordingly, both elements are required for GS biosynthesis (Bones and Rossiter, 1996). The impact of both elements on GS content, especially that of sulfur has frequently been investigated. It turned out that the GS content positively correlates with the sulfur supply. Furthermore, aliphatic GS are the most sensitive to sulfur deficiency, presumably due to the fact that their precursor methionine also contains sulfur, which further increases the effects of sulfur deficiency. The effect of nitrogen on GS accumulation is less clear, and needs to be further studied. Generally, low nitrogen concentrations positively affect GS accumulation, while high nitrogen supplements reduce it (Bones and Rossiter, 1996; Redovnikovic et al., 2008a; Kaur et al., 1990, Falk et al., 2007; Matallana et al., 2006; Alnsour et al., 2012; Omirou et al., 2009; Josefsson, 1970).

Under sulfur limiting conditions, myrosinase activity is increased. This observation led to the suggestion that GS might serve for storing sulfur, which can be remobilized by myrosinase during sulfur deficiency to produce sulfur (Bones and Rossiter, 1996). However, several corresponding studies revealed that GS are not a source for recyclable sulfur in plants, since GS sulfur compose only a small part of plants total sulfur (Redovnikovic et al., 2008a; Rask et al., 2000). Nevertheless, it could not be excluded that under sulfur starvation, GS sulfur may indeed be a significant source for sulfur metabolism.

2.3.4. Glucosinolates and cancer

Several studies conducted on human revealed that the consumption of GS containing plants reduces the cancer risk significantly. The risk of bladder cancer in men could be reduced by the consumption of *Brassica* vegetables by 50%. Moreover, *Brassica* vegetables have been shown to reduce the risk for the colon cancer (Verkerk et al., 2009; Holst and Williamson, 2004).

GS hydrolysis products are thought to play a role in cancer prevention by blocking carcinogens and suppressing the induction of cancer cells. Furthermore, they also induce phase II detoxification enzymes and inhibit phase I enzymes, and they modulate apoptosis and cell cycle perturbations. Finally, they are able of reducing breast and cervical cancer by blocking estrogen receptors (Mithen, 2001; Cartea and Velasco, 2008).

One of the major mechanisms for the chemoprotective activity of isothiocyanates (ITCs) is the elevation of glutathione S-transferase (GST) activity. Experiments with mice, either with lyophilized broccoli as feed or small intestine perfusion of lyophilized broccoli, revealed a significant increase of GST expression. According to the National Cancer Institute in the US, sulforaphane (one of GS hydrolysis products present in broccoli) is one of the most promising anticancerogens (Holst and Williamson, 2004; Johnson, 2003).

In contrast to the results mentioned before, some experiments did not approve a direct link between *Brassica* consumption and cancer risk reduction. Moreover, other studies argued that GS hydrolysis products might have mutagenic and carcinogenic effects (Holst and Williamson, 2004). This contradiction seems to be caused by different doses or concentrations of GS hydrolysis products investigated, since very high concentrations of isothiocynates and nitriles may cause the initiation of carcinogenic, cytotoxic and mutagenic processes (Verkerk et al., 2009).

2.4. Plant metabolism and stress

2.4.1. General aspects

Living organisms are known to metabolize various compounds through their life cycle for maintenance and growth. In principal, two types of metabolites could be distinguished; primary metabolites comprise a few numbers of compounds, which are essential for survival and growth, e.g., amino acids, nucleic acids and sugars (Pichersky and Gang, 2000). In contrast, the so-called secondary plants products are not required for the growth and development of plant cells in general. Accordingly, they are classified as secondary products.

Plant secondary metabolites have received a great interest due to their medicinal, ecological, and nutritional properties. More than 80% of known natural products are from plant origin, a survey of medicinal plant usage among US population shows an enormous increase from only 3% in 1991 to more than 37% in 1998. 75% of the global population uses plants and plant derived substances in traditional medicine (Rao and Ravishankar, 2002). Moreover, in the western countries 25% of the pharmaceuticals are derived from plants. Aspirin is an example: Many plants contain the active molecule acetylsalicylic acid, which nowadays is chemically produced to avoid observed stomachaches side effects (Bourgaud et al., 2001; Rao and Ravishankar, 2002).

Although the role of secondary plant metabolites in internal plant processes has been recognized since the 70´s of the last century, most attention focused on their ecological and medicinal effects and more attention should be given to their role in plant physiology, e.g., as signal molecules. For example, many important signal molecules such as jasmonic acid and salicylic acid were considered as secondary metabolites in the past (D'Auria and Gershenzon, 2005).

Up to now, several hundreds of thousands structures of compounds are known. Apart from the many different basic structures, numerous modifications such as; glycosylation, hydroxylation, methylation, acylation or conjugation to small molecules cause the tremendous diversity and complexity (Pichersky and Gang, 2000; Gachon et al., 2005). Among these modifications, glycosylations are of special interest; beside their role in secondary metabolites stabilization and enhancement of water solubility, sugar molecules

facilitate secondary metabolites compartmentalization (Pichersky and Gang, 2000; Gachon et al., 2005).

In spite of the differences between primary and secondary metabolism, several lines of evidence indicate the connection between primary and secondary metabolism. First, secondary metabolism pathways use substrates derived from primary metabolism. Second, many secondary metabolism pathways use enzymes belonging to primary metabolic pathways, whose functions had been converted by different mutations. Third, secondary metabolism pathways are arranged into clusters, which facilitate gene temporal and spatial expression regulation, using cells regulatory tools (Roze et al., 2011).

It is thought that secondary metabolites pathway enzymes were evolved from primary ones, which interfere somehow in secondary metabolites pathway, further diversification of these genes is achieved by gene duplication, mutation and positive selection. A well documented example for the evolution of a typical secondary metabolite pathway enzyme is the aliphatic glucosinolate chain elongation enzyme in *Arabidopsis* methylthioalkylmalate synthase (MAM) (Vining, 1992; Kroymann, 2011; De Kraker and Gershenzon, 2011).

2.4.2. Plant stress

In contrast to animals, plants cannot change their location. Accordingly, they are much more affected by environmental impacts. Consequently, plants are frequently subjected to various biotic and abiotic stresses, and they have to alleviate these stresses. McCue and Hanson (1990) classified plant resistance to stresses into four levels of organization; metabolic, physiological, structural and developmental (McCue and Hanson, 1990; Knight and Knight, 2001; Fujita et al., 2006; Hussain et al., 2011).

Stress can be defined as adverse conditions that inhibit a biological system, e.g., plant from well being and normal functioning. Such conditions will prevent plants from reaching their genetic potential, causing yield losses. Abiotic stresses, primarily drought and salinity, are the major causes of plant stress and related crop loss. Drought stress and salinity are responsible for more than 50% of crop loss worldwide, causing the loss of hundreds of millions of dollars and endangering the agriculture industry. Moreover, it is proposed that 50% of the arable land will suffer from salinity by the year 2050 (Knight et al., 1997; Wang et al., 2003; Mahajan and Tuteja, 2005; Hussain et al., 2011).

Water stress can occur in two forms, either by excess or shortage of water supply. In the case of excess water supply, negative effects are caused by reducing O_2 availability to plant roots causing suffocation. More common is drought stress, characterized by limited water availability, frequently occurring in arid and semiarid regions, which compose one third of the world´s land area. Drought can be classified as permanent, seasonal and random drought. In addition to these, non-apparent drought might occur, when transpiration rate due to hot weather and wind increases and more water is transpirated than can be absorbed by the roots, despite adequate soil water content (Díaz et al., 2005b; Mahajan and Tuteja, 2005).

As plant cannot physically avoid drought stress due to their immobility, they have developed several strategies to adapt to such adverse conditions. Many plants escape the drought, like annual plants, which grow and prosper only in periods of water availability. Another strategy is the adaptation to drought anatomically and morphologically, e.g., thick cuticle layer of shoot and deep root system as well as the ability to close the stomata (Díaz et al, 2005b). Drought causes cells dehydration and osmotic imbalance. Cells suffering from drought stress show increased membrane porosity and permeability, increased protein denaturation and accumulation of cellular electrolytes (Díaz et al., 2005b; Mahajan and Tuteja, 2005). However, the main drawback of drought stress induced stomata closure is the reduction of CO_2 supply for photosynthesis, which causes massive imbalances in the reduction status (Wilhelm and Selmar, 2011).

Soil salinity is majorly caused by increased concentrations of NaCl, which beside the adverse effects of elevated Na^+ and Cl^- concentrations in the cells, affect K^+ and Ca^{2+} concentrations in the plant cells causing disruption of ionic homeostasis in the cell. Osmotic imbalance inside and outside the cells also affect water and nutrients absorption by the root (Cramer et al., 1987; Knight et al., 1997; Díaz et al., 2005b). Moreover, due to the reduced water uptake, also the typical drought stress symptoms are induced.

2.4.3. Plant response to abiotic stress

Plant responses to abiotic stresses range from accumulation of compatible osmolytes and proteins, and hormone homeostasis to morphological changes in foliage. Under stress conditions, cells expansion is reduced resulting in smaller leaves. Moreover, under water stress roots show reduced mitotic activity (Schuppler et al., 1998; Wang et al., 2003; Vollenweider and Günthardt-Goerg, 2005; Hussain et al., 2011).

Drought and osmotic stress lead to stomata closure, which reduces water loss by transpiration, but causes massive reductions in CO_2 availability. Under such conditions, the generation of superoxide radicals is enhanced (Mehler reaction) (Díaz et al, 2005b). In order to prevent the production of toxic oxygen species, various protecting mechanisms, e.g., non photochemical quenching, have been evolved.

In contrast to laboratory conditions, where different abiotic stresses can be applied seperately, in natural environments generally, different types of stresses occur simultaneously (Selmar and Kleinwächter, 2013). Moreover, due to various crosstalks of signaling pathways, the manifestation of a resistance to different abiotic stresses is very complex (Fujita et al., 2006).

2.4.3.1. Biochemical responses to osmotic stress and suitable marker selection

In response to different osmotic stresses, a wide variety of metabolites is synthesized, usually different metabolites are accumulated in response to different stresses. Amino acids, sugars, reactive oxygen radicals, proteins and compatible solutes are known to accumulate under different stress conditions. Compatible solutes, such as carbohydrates, polyamines, organic acids and amino acids, accumulate to high concentrations in the cell. It is of great importance to choose a suitable stress indicator in order to accurately identify plant stress status (McCue and Hanson, 1990; Díaz et al., 2005b).

One of the most prominent compounds accumulated in response to various stress situations is the non-proteinogenic amino acid γ-aminobutyric acid (GABA). It is found in bacteria, vertebrates and plants. In plants GABA is thought to play a role in pH regulation, nitrogen storage, or to be part of signaling pathways (Shelp et al., 1999; Bouche and Fromm, 2004).

GABA is the most used marker for drought stress. Moreover, it was found to accumulate in response to other stress conditions (Selmar and Kleinwächter, 2013). Salt stress is quite similar to drought stress in that both types cause osmotic stress. Therefore GABA was expected to accumulate in response to salt stresses as well. Indeed, it was recently found that GABA is accumulated in *Nicotiana sylvestris* plants subjected to 100 mM NaCl (Akcay et al., 2012).

2.4.4. Plant stress signaling

In signaling pathways of plants challenged by biotic and abiotic stress many hormones are involved, especially the involvement of abscisic acid, jasmonic acid, ethylene and salicylic acid had been reported (Fujita et al., 2006).

Abscisic acid (ABA) is a plant hormone responsible for seed dormancy and stomata closure, e.g., ABA is also responsible for the delay of seed germination until favorable conditions are present.

The ABA concentration increases after stress treatments. Moreover, the genetic expression patterns in response to osmotic stress -caused by drought, salinity and cold- is similar to that after ABA application. Also similar to stress conditions, ABA applications cause cell desiccation and osmotic imbalance (Leung and Giraudat, 1998; Xiong and Zhu, 2001; Finkelstein et al., 2002; Sharp, 2002; Sharp and LeNoble, 2002; Mahajan and Tuteja, 2005).

Studies suggest that cell metabolism and genetic expression responses to osmotic stress are transmitted through at least two different pathways; an ABA dependent and an ABA independent pathway. Signaling components of both pathways crosstalk or even converge with each other. Ca^{2+} concentrations are increased in response to ABA and osmotic stress. Therefore, calcium is suggested to play a major role in crosstalk of different stress signaling pathways (Koornneef el al., 1998; Leung and Giraudat, 1998; Knight and Knight, 2001; Mahajan and Tuteja, 2005).

Salicylic acid (SA) is a well-known plant signaling molecule (Blackman and Davies, 1985; Raskin, 1992; Sharp, 2002; Fujita et al., 2006). SA effects plant flowering, it plays a role in alleopathic and thermogenic reactions, and functions in the signaling of plant resistance against pathogens (Raskin, 1992). Exogenously applied SA causes increased plant tolerance to many abiotic stresses, such as chilling, drought and salt stress (Sawada et al., 2008; Arfan et al, 2007), and plays a predominant role in systemic acquired resistance (Hayat et al., 2010).

Jasmonic acid (JA) is involved in plants interaction with their environment. JA affects fruit ripening, leaf senescence, root growth and pollen development. Moreover, it is a signal compound within the defense system against insects and pathogens (Rohwer and Erwin, 2008; Mikkelsen et al., 2003). Methyl jasmonate (MeJA) emitted from wounded plants induces resistance in adjacent non-wounded plants (Rohwer and Erwin, 2008).

JA is thought also to play a role in abiotic stress responses, as corresponding genes were stimulated following JA and drought treatments. Moreover, in soy bean leaves subjected to dehydration, JA content increased rapidly after 2 hours, although its concentration decreased later (Creelman & Mullet, 1995).

The phytohormone ethylene has many functions in plants, e.g., it regulates leaf and flower senescence, hairy root initiation, seed germination and fruit ripening. Apart from these effects on developmental processes, it also controls many plant responses to a wide variety of stresses (Tanaka et al., 2005). Moreover, ethylene is part of the crosstalk with ABA to control stomata closure and plant development (Tanaka et al., 2005; Chaves et al., 2003).

2.4.5. Stress and plant secondary metabolites

Plant secondary metabolites have frequently been investigated for their pharmaceutical and ecological roles. In *Brassica* crops and the model plant *Arabidopsis thaliana*, the glucosinolate-myrosinase system and its role as defense system has been thoroughly studied. However, many observations suggest also other roles of this group of secondary plant metabolites (DÁura and Gershenzon, 2010).

A simple observation of higher quality of condiments and spices produced in arid and semi-arid regions indicated the presence of higher concentrations of the corresponding secondary metabolites. Indeed, many classes of secondary plant metabolites show an increase in their concentrations when subjected to abiotic stresses, examples include phenols, essential oils, alkaloids and GS (Selmar and Kleinwächter, 2013; Gershenzon, 1984).

Studies carried out to investigate the effect of methyl jasmonate and salicylic acid on GS contents in *Arabidopsis*, resulted in low or no increase of aliphatic GS contents. As explanation for this finding the authors suggested that aliphatic GS in *Arabidopsis* are constitutively regulated. The intersection of indole glucosinolate biosynthesis pathway with indole acetic acid (IAA) biosynthesis suggested a role of GS, although indirect, in plant development (Mikkelsen et al., 2003; Bender and Celenza, 2008). Thus, it is evident that secondary plant metabolites in general and GS in particular have different so called functions than the well defined plant-insect/pathogen interactions.

2.4.5.1. Over-reduced status

As mentioned before, osmotic stress induces many reactions in the plant to alleviate the stress. One common reaction to mitigate restricted water absorption, either due to water scarcity or ions imbalance, is stomata closure to minimize water loss. However, closed stomata reduce also the CO_2 influx to the plant, which greatly affects the photosynthesis machinery (Selmar and Kleinwächter, 2013).

Under normal conditions, photosynthetically produced energy, stored in the form of $NADPH+H^+$ and ATP exceeds the plant's demand for CO_2 fixation. Consequently, plants produce more $NADPH+H^+$ than they can consume in the Calvin cycle. Accordingly, the electron transport chain delivers much more electrons than could be transferred to $NADP^+$. As result, electrons could be transferred to oxygen, generating toxic superoxide radicals. Plants have evolved many mechanisms to abolish this oversupply of energy and to prevent the production of oxygen radicals, for example, non photochemical quenching, re-oxidation of $NADPH+H^+$ by xanthophyll cycle and photorespiration. Moreover, superoxide radicals generated could be detoxified by the superoxide dismutase and ascorbate peroxidase systems (Wilhelm and Selmar, 2011; Selmar and Kleinwächter, 2013; Bohnert and Sheveleva, 1998).

Whereas under regular conditions the various mechanisms for energy dissipation suppress the generation of superoxide radicals, the situation under drought stress changes drastically. Due to stomata closure and CO_2 limitation, dark reactions of the photosynthesis decrease strongly. Thus, a bigger pool of reducing power is accumulated as the ratio of $NADPH+H^+$ to $NADP^+$ increases. According to the law of mass action, all reactions shift to reduction side of equilibrium when the reductive power is enhanced. Thus, the production of highly reduced secondary plant metabolites must be increased, taking into account no enzymatic activities are changed. The CO_2 concentration effect on secondary plant product accumulation was elegantly presented by Nowak et al. (2010). As expected, monoterpene levels increased in *Salvia officinalis* plants subjected to drought stress (closed stomata and lower CO_2 levels), and were lowered when CO_2 concentration was increased (Selmar and Kleinwächter, 2013; Nowak et al., 2010).

2.4.5.2. Effect of abiotic stress on secondary plant metabolites

Accumulation of secondary plant metabolites in response to environmental factors has been reviewed by Gershenzon (1984), and more recently by Falk et al. (2007) and Selmar and Kleinwächter (2013). Most classes of secondary plant metabolites are increased in their concentrations under drought stress. However, due to lower biomass of stress treated plants, often it is not clear if the increase in concentration reflects an enhanced synthesis of these metabolites, or if it reflects a concentration effect in response to reduced plant biomass (Gershenzon, 1984; Selmar and Kleinwächter, 2013). Only in few studies a real increase in the total amount of the secondary plant products in response to stress is well documented.

One example represents the work by Nowak et al. (2010), who showed that monoterpene concentrations in *Salvia officinalis* increase in plants subjected to moderate drought stress. The increase was sufficient to compensate the drought-related biomass decrease, resulting in higher total amounts of monoterpene than the well watered plants. However, other authors reported that terpenoids and monoterpene total contents decrease in *Melissa officinalis*, *Salivia officinalis* and *Nepeta cataria* (Manukyan, 2011).

The enhanced concentrations of phenolic compounds and anthocyanins in drought stressed plants resulted in total increase of these products, ranging from 10-25% in comparison with the control plants. While, differences in flavanoid and furoquinone concentrations in drought treated plants led to similar or lower total contents of these metabolites in comparison to the well watered control plants (Selmar and Kleinwächter, 2013).

2.4.5.3. Effect of abiotic factors on glucosinolate accumulation

In various studies, genetic factors had been found to greatly affect GS accumulation, different cultivars of *A. rusticana* and *B. rapa* not only contain different total GS concentrations, but also individual GS concentrations differed as well (Kang et al, 2006; Li and Kushad, 2004). Different plant organs and developmental stages reveal a great effect on GS concentrations in *A. thaliana* plants (Brown et al., 2003). In addition, environmental factors play an important role in GS accumulation, Engelen-Eigles et al. (2006) showed that gluconasturtiin concentration in watercress was positively affected in response to lower temperatures, long days (long photoperiod), and light quality.

Due to nitrogen and sulfur presence in GS structure, it is supposed that these nutrients will affect GS accumulation in plants. In contrast to nitrogen, sulfur is known to affect markedly accumulation of GS (Falk et al., 2007); total glucosinolates increased in *in vitro* grown *T. majus* and *A. rusticana* plants (Mattallan et al., 2006; Alnsour et al., 2012). In hydroponic grown *Brassica campestris* (Hu et al., 2011; Xin-Juan et al., 2006), green house grown *Brassica rapa* (Kim et al., 2002) and *Brassica oleracea* (broccoli) grown in field (Schonhof et al., 2007).

Little information is known how abiotic stress impacts GS concentration and/or content in plants. Most work on stress effects on GS accumulation in plants has been done with regard to biotic stress caused by insects. Corresponding plant-insect relations were recently reviewed by Hopkins et al. (2009). In the following passage, a brief review of relevant works on the impact of abiotic factors on GS accumulation in plants is compiled.

Two studies reported a massive increase in GS concentrations in *Brassica napus* plants subjected to drought stress (Champoiliver and Merrien, 1996; Jensen et al., 1996). Total, aliphatic and indole GS content in *Arabidopsis* increased in the first five days when plants were subjected to drought stress induced by polyethylene glycol treatment. However, after day five the content of these GS decreased to levels lower than that of control plants (Jing et al., 2010).

In response to salt stress radish (*Raphanus sativus*) sprouts accumulate less GS at lower NaCl concentrations, while higher NaCl concentrations promoted GS accumulation when compared with control treatments (Yuan et al., 2010). 50 mM NaCl treatment of *B. campestris* grown in greenhouse resulted in increase of total, indole and aliphatic GS, while aromatic GS remained constant. When NaCl was increased to 100 mM, total, indole and aliphatic GS decreased but remained higher than concentrations in control plants, aromatic GS decreased to almost the half of control plants GS concentration (Keling and Zhujun, 2010).

Tryptophan metabolizing enzymes CYP79B2 and CYP79B3 were upregulated and the amount of indole GS was increased in *A. thaliana* treated with methyl jasmonate (MeJA) (Mikkelsen et al., 2003). *B. napus* leaves revealed higher gluconasturtiin concentrations than control plants, when salicylic acid (SA) was added to the soil (Kiddle et al., 1994).

From the data presented so far on the impact of abiotic factors on GS concentration, it could be deduced that these factors indeed effect GS accumulation. Unfortunately, some of the results of these investigations are contradictory. Moreover, most of them did not consider the treatment effect on the plant fresh weight and/or dry matter percentage. Furthermore, no or only few investigations addressed whether the increase in GS concentrations actually reflects higher GS contents, or if it is due to smaller plant size, usually correlated with stress.

Especially with respect to horseradish, comprehensive studies investigating the GS accumulation in root tissues in response to different abiotic factors are required, which also consider the major storage roots with its high economical value. Finally, there was no study investigating different abiotic factors effect on GS accumulation in a single species at different cultivation systems. Therefore, these studies will contribute to elucidate the complex issue of plant secondary metabolism in response to abiotic factors.

3. Materials and Methods

3.1. Plant material

3.1.1. Plant cultivation

Planting materials, secondary roots or "sets" as they usually are called, of horseradish (*Armoracia rusticana* Gaertn., Mey. & Scherb.) were obtained from two commercial plantations. The first is located in Eastern Germany (Gemüsebaubetrieb Spreewald, Lübbenau); in this thesis referred to as Eastern variety (E). The second provider (Kräuterhof Funke, Adelsdorf-Neuhaus) is located in Southern Germany, this grower supplied us with two different varieties Kröner and Badisch. They were designated by these names or initials as K and B.

Planting materials had been stored as described for commercial plantations: Horseradish sets received in late winter were stored in pits. The sets were kept there until the beginning of spring. The sets started to form sprouts and some leaves, when the weather became suitable for growing the plants (end of April-beginning of May). Sprouting plants were taken out of the soil, prepared for planting (see below) and restored in the pits, and planted 24-48 hours after sets preparation (Figure 6; Courter and Rhodes, 1969; Kadow and Anderson, 1940; Bratsch, 2009).

Figure 6: Sets storage, after preparation and before planting.

After the sets were digged out from the pits, they were briefly cleaned and transferred to the lab for further processing. Only healthy sets free of black lesions or hollowness were used. Length of chosen sets was adjusted to 25±1 cm, with square cut at the top and slant cut at the bottom to mark planting direction. All sprouts and leaves had been stripped, except 1-3 sprouts at the upper edge of the sets to ensure good root quality and to minimize multiple sprouting. (Courter and Rhodes, 1969; Kadow and Anderson, 1940; Bratsch, 2009). In order to manage the experimental design and data analysis later on, each individual root (set) was weighed and uniquely labeled with a serial number and the variety name (Figure 7).

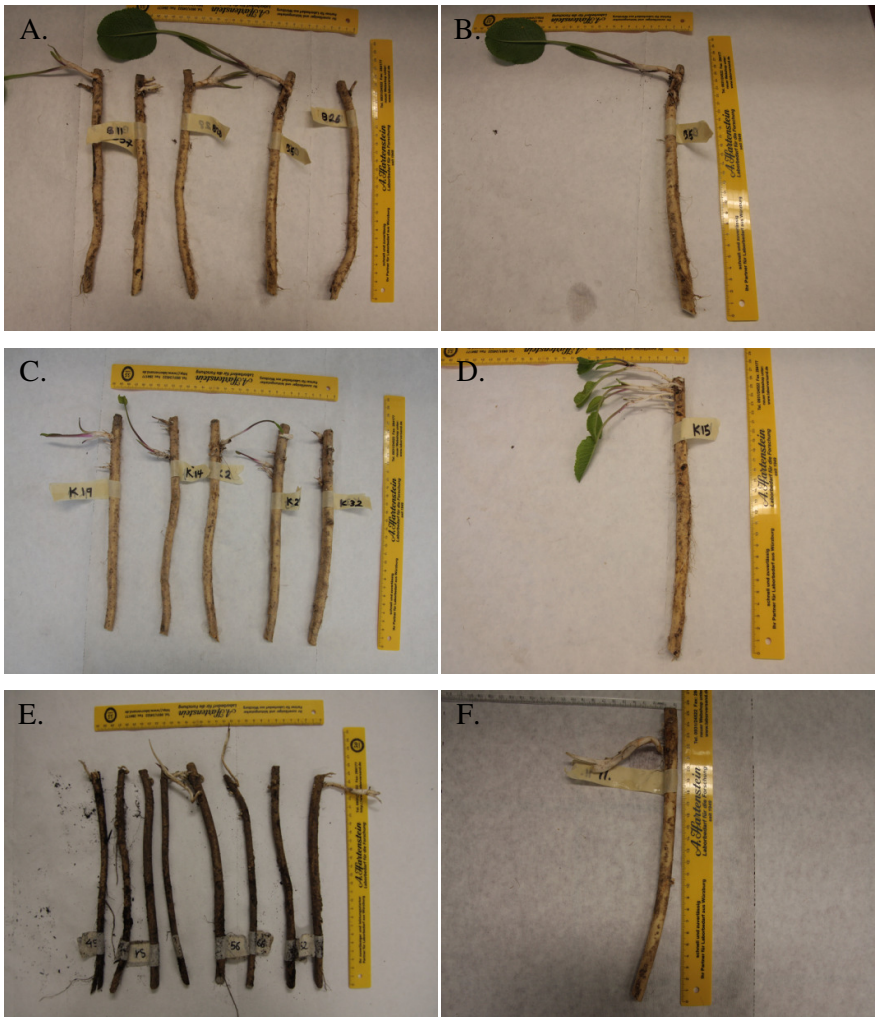


Figure 7: Sets preparation for planting in different experiments. A and B from Badisch, C and D from Kröner, E and F from Eastern varieties.

3.1.2. Plants harvest and sample preparation

3.1.2.1. Plants harvest

Plants were grown until end of fall and beginning of early winter. After almost all leaves were senescent due to the first frost (Ravindran and Pillai, 2004), the roots were harvested. Harvest was performed manually in order to minimize injuries and for solid determination of plant weight. For this, plants were briefly cleaned at the place of plantation, labeled and then transferred to the lab.

In the lab, further cleaning of the plants roots was performed with tap water. Then the roots were blotted on tissue paper and dried, and their weights were recorded. Then, homogenous samples of each plant were taken for biochemical analysis and dry weight determination. Plants grown in the botanical garden (BG) of TU Braunschweig and rain shelter (RS) at the Institute for plant biology were harvested in the same manner.

3.1.2.2. Sampling and sample preparation

Homogenous samples from roots of individual plants and individual leaves from the same plant (when needed) were taken. Samples from main and secondary roots of each plant were taken 5 cm below the attachment region of shoots and roots. Two representative samples were taken from roots of each plant. The first one was directly shock frozen in liquid nitrogen and stored at -80 °C until being further processed. The other one was used to determine the dry weight (Figure 8).

Figure 8: sampling of *A. rusticana* roots for biochemical analysis and dry weight determination.

Frozen samples were crushed in a pre-cooled mortar with a pistil under liquid nitrogen to small pieces ≈3-5 mm. A ball mill (Retsch MM200) was used to grind the material to fine powder (two times 1 minute each at 25 Hz S^{-1}), which then was further lyophilized. Dried material was stored at -20 °C until being used for biochemical analysis. Individual root samples obtained from individual plants were used for GS determination. Mixed root samples were used to determine GABA concentration and myrosinase activity. For this, about 200±10 mg dried material from each plant was exactly weighed. These mixed samples were used for GABA and myrosinase analysis.

Homogenous leaf samples of different treatments at the RS were taken from all viable leaves using a cork borer (diameter, 2 cm). These samples were used to determine dry weight and for biochemical analysis. To determine GS concentrations in different treatments at the RS, one leaf disk (diameter, 2 cm) was taken from each viable leaf of the plant. In addition, the previously described procedure was also used for the leaf sample collection for DW determination. In order to determine GS concentrations in different leaves of the same plant, leaf disks of individual leaves were taken.

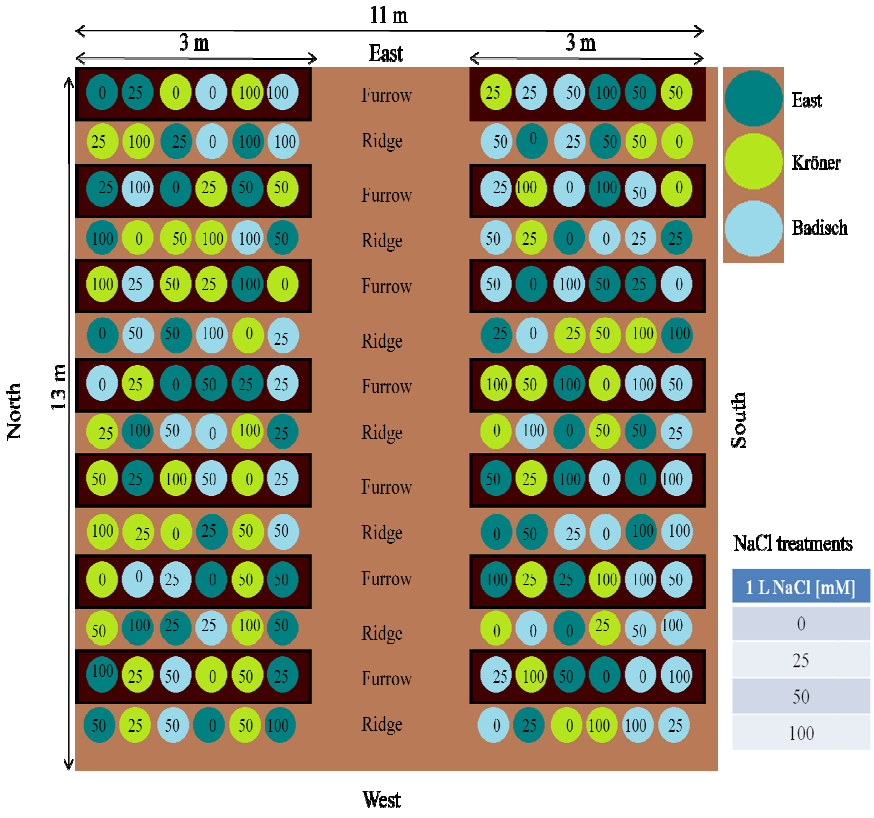


Figure 9: Treatments random distribution in the field.

3.1.3. Experimental treatments

3.1.3.1. Drought and salinity experiments in field

Eight different treatments were planned. Each treatment was conducted with seven plants from each variety for the three different varieties with a total of 168 plants, these treatments were two (different soil) water contents caused by the field design as well as four NaCl (0.0, 25.0, 50.0 and 100.0 mM) treatments (Keling and Zhujun, 2010). Different treatments were randomly distributed in the field (Figure 9).

3.1.3.2. Drought experiment in rain shelter

Plants originating from the three varieties were used, and four different watering treatments were planned. Each treatment was applied on 10 plants. Thus, 40 plants from each plantation were used, and 120 plants were used in the total experiment.

3.1.3.3. Salicylic acid experiment in rain shelter

Sets prepared as previously described, were used for the experiment elucidating the influence of salicylic acid. The plants were grown in a rain shelter. Seven plants of the East variety were grown for each treatment of salicylic acid, and left until they were fully developed. Then salicylic acid treatments were applied. Identical pots containing the same amount of commercial potting soil (Compo Sana) were used (Figure 10).

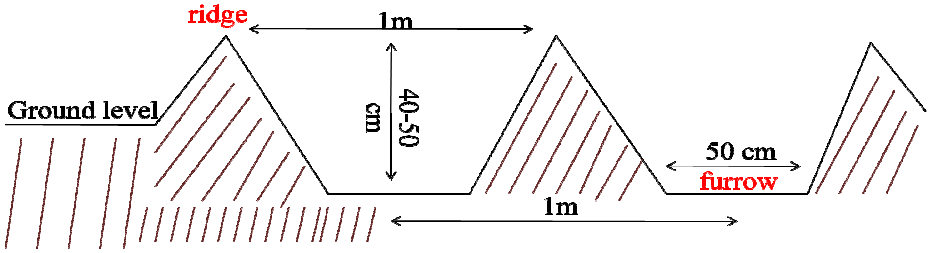
Figure 10: Horseradish plants planted for salicylic acid experiment in the rainshelter.

3.1.4. Site preparation and planting

3.1.4.1. Field preparation and planting

Field experiment was conducted in 2010 at the botanical garden of TU Braunschweig. The entire area was divided into two parts to facilitate maintenance practices. The field was prepared and plants were grown as described for commercial plantations with some modifications (Figure 11): In order to generate distinct differences in water supply, furrows (3 m long and 50-60 cm wide) were established, and corresponding ridges were raised. The difference of height between the surface of the two soil designs was 40-50 cm (figure 11, A). Sets were led in wholes at 45^{o} angle and covered by soil. Plants were grown 50 cm apart, and 1 m between different rows (ridge-ridge or furrow-furrow).

A.



B.

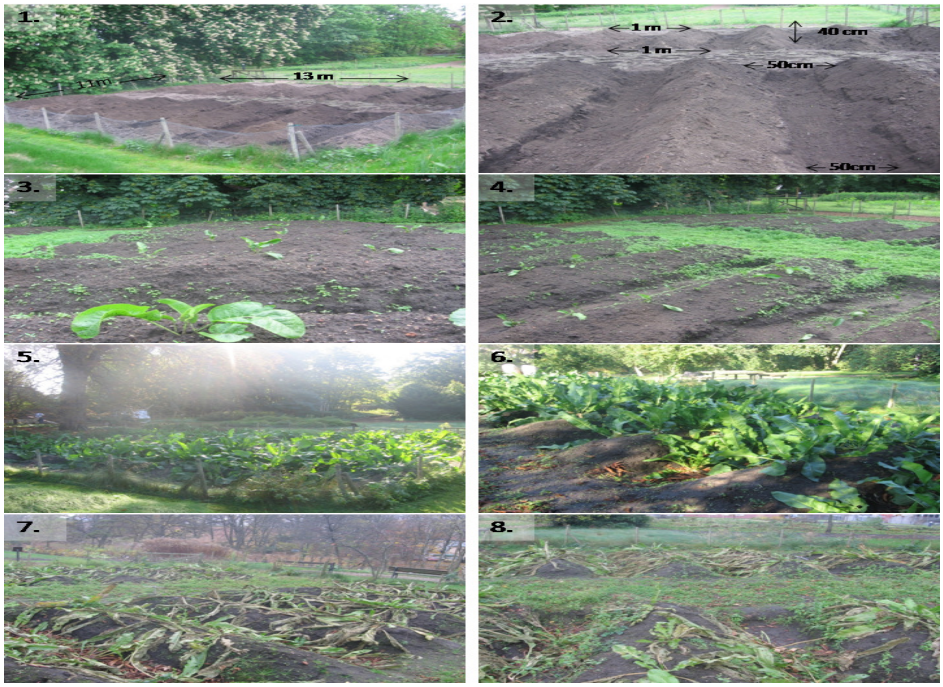


Figure 11: Field preparation sketch (A). (B) shows stages from field preparation and cultivation (1 and 2), plants early growth (3 and 4), plants optimum growth (5 and 6) and at the end of season (7 and 8).

3.1.4.2. Rain shelter location preparation and planting

To avoid water supply by rain, plants were grown under rain shelter. This rain shelter protected the plants from rainfall and at the same time plants were allowed to grow under normal temperature and humidity conditions. Controlled watering was performed by applying exactly the required amount of water. The construction of this rain shelter is presented in (Figure 12). Labeled sets were planted in spring of 2010 in predetermined pots.

Figure 12: Constructing a suitable plant shelter to investigate drought effect on glucosinolate accumulation in *A. rusticana.*

3.1.5. Humidity measurements

3.1.5.1. Humidity measurements in field

Water contents of the furrows and ridges were measured every week using TRIME-FM mobile moisture meter with an appropriate probe (p3z). Measurements were carried on according to the schedule presented in Figure 13. After completing scheduled measurements, new series of measurements started from the beginning.

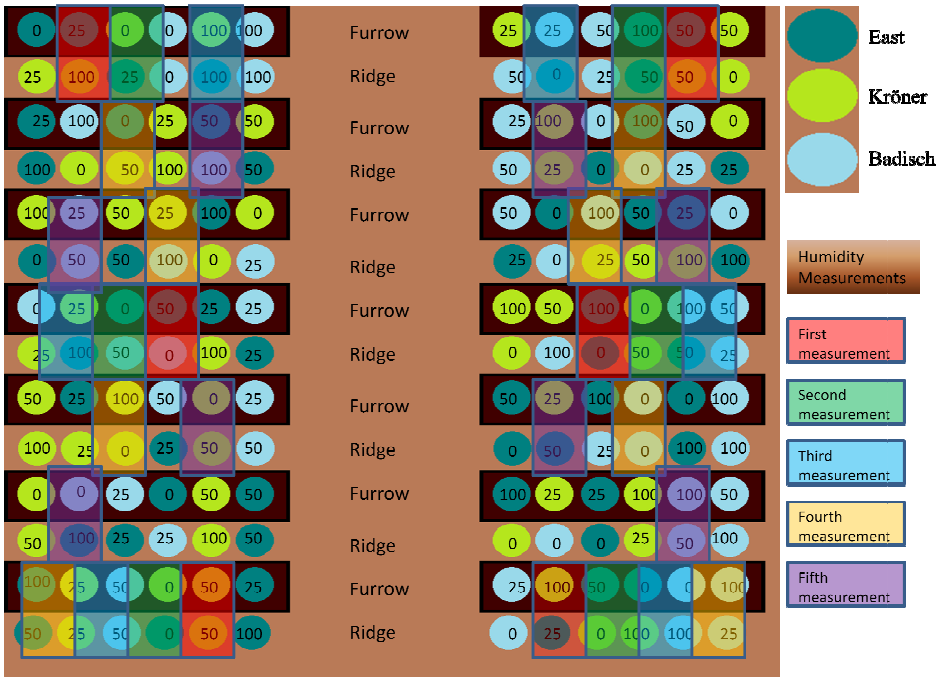


Figure 13: Schedule for humidity and temperature measurements in the field.

3.1.5.2. Plants irrigation and water content measurements in rain shelter

3.1.5.2.1. Water holding capacity determination

Standard commercially available potting soils (Compo Sana), assigned to be used in growing plants were mixed in a greenhouse until used later. Water holding capacity (WHC) of the potting soil was determined by two ways. Firstly, sample dry matter was determined by using an infrared heater with analytical balance (Ultra-X 210, Gronert, Germany). Then, 100 g fresh material was weighed and placed in an apparatus that allows water movement to surrounding sand, and the whole system was weighed. Soil sample was saturated with water overnight and weighed at different intervals until it stopped losing water or lost it at very slow rate about 6 hours after saturation. WHC was calculated from soil wet and dry weight.

Secondly, several holes were drilled in the bottom of two pots identical to those used in the experiment. Then they were filled exactly with the same amount of potting soil (4 kg) and saturated with water. Pots were weighed at different time interval till 8 hours.

3.1.5.2.2. Plants irrigation and water content measurements

For the growing experiment under the rain shelter, identical commercial pots (volume = 10 L) with closed bottoms were purchased in order to make sure that defined amounts of water

were used, and there is no loss through percolation. Each pot was weighed and filled with the same amount of soil (4 kg).

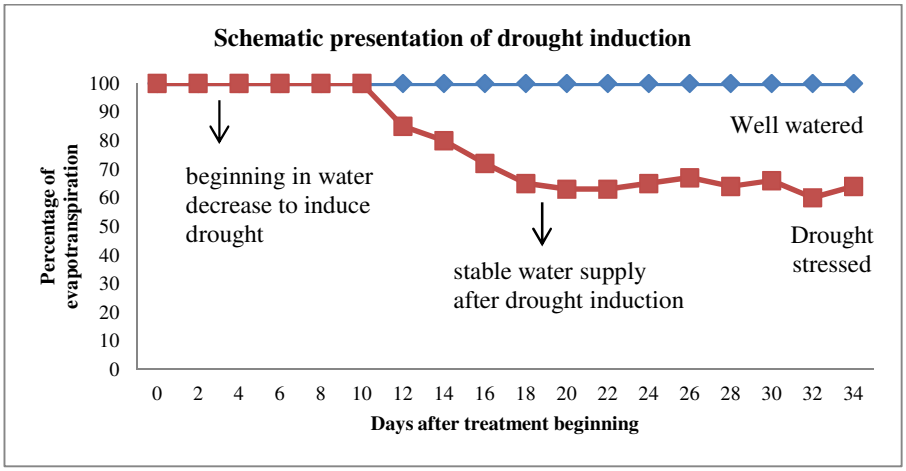


Figure 14: Schematic presentation of drought stress induction in horseradish plants grown in the rain shelter.

All plants were well watered until they have reached their full size. In order to induce drought stress, the amounts of irrigated water was reduced. Plants receiving water amounts corresponding to WHC were irrigated with water amounts, which corresponded to the amount of water lost by evapotranspiration. Thus, a constant water amount was preserved. Plants in other treatments were receiving less water amounts than they lost by evapotranspiration. To induce different degrees of drought treatments, water supply was differentially decreased. This procedure continued until different rates of evapotranspiration were achieved. Surprisingly, water reduction did not cause a corresponding reduction in evapotranspiration of the second treatment, which indicates that the plants were receiving sufficient amounts of water, compared to plants receiving water amounts equal to WHC. However, further water reduction in the third and fourth treatment resulted in corresponding decreases in evapotranspiration. The decreases in the evapotranspiration rate were different in response to different treatments and resembled different degrees of drought stress. A schematic presentation of drought induction is presented in Figure 14.

After reaching the required water content in the pots of each treatment, individual pots were weighed twice a week, and the amount of used and/or lost water by evapotranspiration was added manually to reset the plants to the same water conditions through the experiment. Water contents in different pots were measured two times a week by TRIME-FM mobile moisture meter (p3z) probe.

3.1.5.2.3. Transpiration measurements

In order to investigate whether different water treatments have affected plants physiology, and whether plants have responded by closing their stomata, which indicates stress induction. One plant variety was chosen and 3 plants from each treatment were used to measure transpiration. Measurements were done by weighing different pots and determining amount of lost water under the conditions of the treatments.

3.1.6. Plants maintenance and fertilization

3.1.6.1. Plants maintenance and fertilization in the field

As insects and pathogens have not been a serious problem, there was almost no need for any practice to control them. In contrast, weeds represented a big and continuous problem and were manually removed.

In order to provide optimal growth conditions, sulfur content in the soil was analyzed prior to planting (appendix, Table A1). Homogenous samples from furrows and ridges were taken from many sites of the field. Sulfur amendment (36 kg/ha) was applied in the form of high sulfur slow dissolving commercial fertilizer (COMPO-NTEC; 14% N, 10% S). Plants requirements of fertilizer were calculated to be 9 grams/plant. Accordingly, 4.5 g fertilizer was applied two times, to maintain sulfur availability throughout the period of plant growth. Due to a dry weather period in 2010 at early growth stages, young seedlings were suffering from severe drought. Therefore, two supplementary irrigations with a sprinkler were applied.

3.1.6.2. Plants maintenance and fertilization in rain shelter

Commercial (high sulfur) fertilizer (COMPO-NTEC; 14% N, 10% S) was used in the same manner as used for Brassicaceae crops, plant requirement was calculated to be 9.0 g fertilizer/ plant. The fertilizer (4.5 g) was applied twice to ensure sufficient sulfur supply throughout the experiment. First supplementary fertilization was applied according to the potting soil producer 6-8 weeks after planting, and the second time 6 weeks after the first supplementary fertilization.

Brassicaceae crops are known to be attacked by several specialist and generalist insects and numerous pathogens. However, as pesticides are supposed to affect plant reaction and thereby GS accumulation, plants grown in RS were checked twice a day, and a tedious manual

collection of insects was practiced two times daily in the morning and evening. Weeds were efficiently manually removed.

3.1.7. Treatments application

3.1.7.1. Treatments application in the field

Plants were continuously subjected to different water amounts due to the field design. Additionally, NaCl was manually added by a concentrated solution to the plant crown region. Different volumes of concentrated solution resembling 250 ml of 0.0, 25.0, 50.0, and 100.0 mM NaCl were added 4 times. Thus, each plant received 1 L NaCl of the concentrations mentioned earlier.

3.1.7.2. Treatments application in rain shelter

3.1.7.2.1. Drought experiment

All sets planted (in labeled pots) were well watered manually until they reached full size about 6 weeks after planting (Figure 15, B & C). Then, plants were irrigated with different water amounts until the required water content was achieved. Pots were weighed and water loss was compensated manually to maintain constant water contents during the experiment.

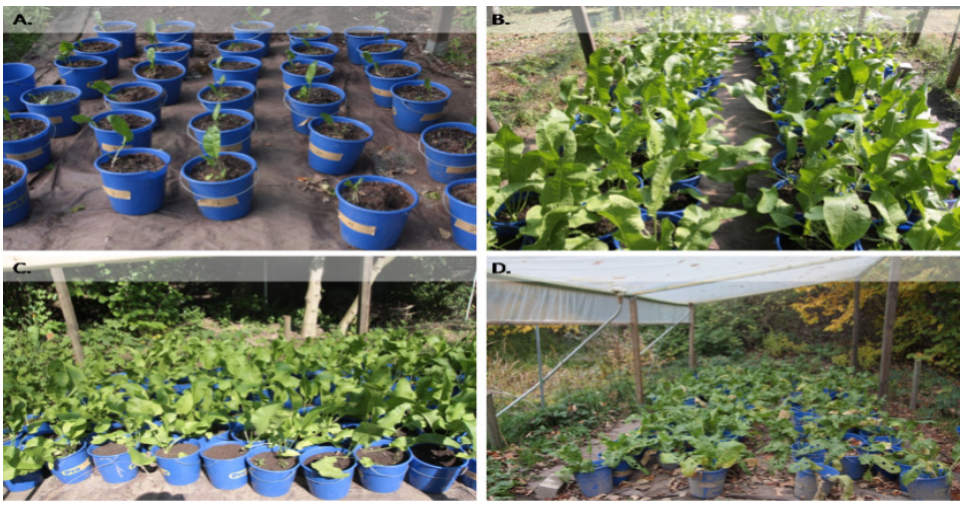


Figure 15: Different stages of plant growth in RS grown plants. Early growth (A), optimum plant growth (B and C), and at the season end (D).

3.1.7.2.2. Salicylic acid experiment

1L of different Salicylic acid (SA) solutions was freshly prepared by dissolving the required amount of SA in few drops of ethanol (96%): Then, the volume was adjusted to 900 ml using H_2O. 1 N KOH was used to set the solution to pH 7.0 before the volume was set to 1L by H_2O (Gutierrez-Coronado et al., 1998; Elwan and El-Hamahmy, 2009). Treated plants were well irrigated. 100 ml of 1.0 or 5.0 mM SA were applied to the potting soil. 100 ml water containing the same volume of ethanol used to dissolve the SA was used to irrigate the control plants (Kiddle et al., 1994). Then, plants were left 5-7 days without irrigation. The salicylic acid treatments were applied for 4 times.

3.2. Culture of *in vitro* plants

3.2.1. *A. rusticana in vitro* plants: Line initiation, regeneration and growth optimization

Initial attempts to germinate *A. rusticana* seeds obtained from two seeds collection in Germany and France failed. Therefore, direct regeneration of *in vitro* plants from *A. rusticana* leaves as described by Pawelczak et al. (2006) was applied. However, also this attempt was not successful.

Alternatively, regeneration of *in vitro* plants derived from calli was used. Callus was formed from mature *A. rusticana* leaves: Explants were cultured on MS media supplemented with 1.0 mg /L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/ L benzyl adenine (BA). *A. rusticana in vitro* plants were regenerated directly from the formed callus by transferring it on MS media supplemented with 0.5 mg /L 1-Naphthaleneacetic acid (NAA) and 0.1 mg /L BA (Table 1; Pawelczak et al., 2006; Araki et al., 1995).

Table 1: Media composition and phytohormone supplementations used for callus formation, plant regeneration and plant growth optimization.

Experiment	Medium	Phytohormone
Callus formation	MS	1.0 mg /L 2,4-D 0.5 mg/L BA
Plant regeneration	MS	0.5 mg /L NAA 0.1mg /L BA
Plant growth optimization 1	MS	0.0, 0.005 and 0.05 mg /L BA 0.5 mg/L NAA
Plant growth optimization 2	½ MS	0.5 mg/L NAA
	MS	……..

For regeneration of plants, phytohormone levels were changed as described in Table 1. Regenerated plants were further transferred to two media to optimize root formation and plant growth. First, regenerated plants were transferred to MS media supplemented with different concentrations of BA (0.0, 0.005 and 0.05 mg/ L) to optimize plant and root growth. Later on, these plants were transferred to MS media lacking NAA or containing half concentration of MS media supplemented with 0.5 mg L/ NAA (Table 1; Pawelczak et al., 2006). In order to obtain plants with identical genetic material, one single plant line was chosen and plants were propagated asexually to obtain the required number of identical plants, which were later used in different experiments. Figure 16 shows different steps of *in vitro* plants line initiation and their growth optimization.

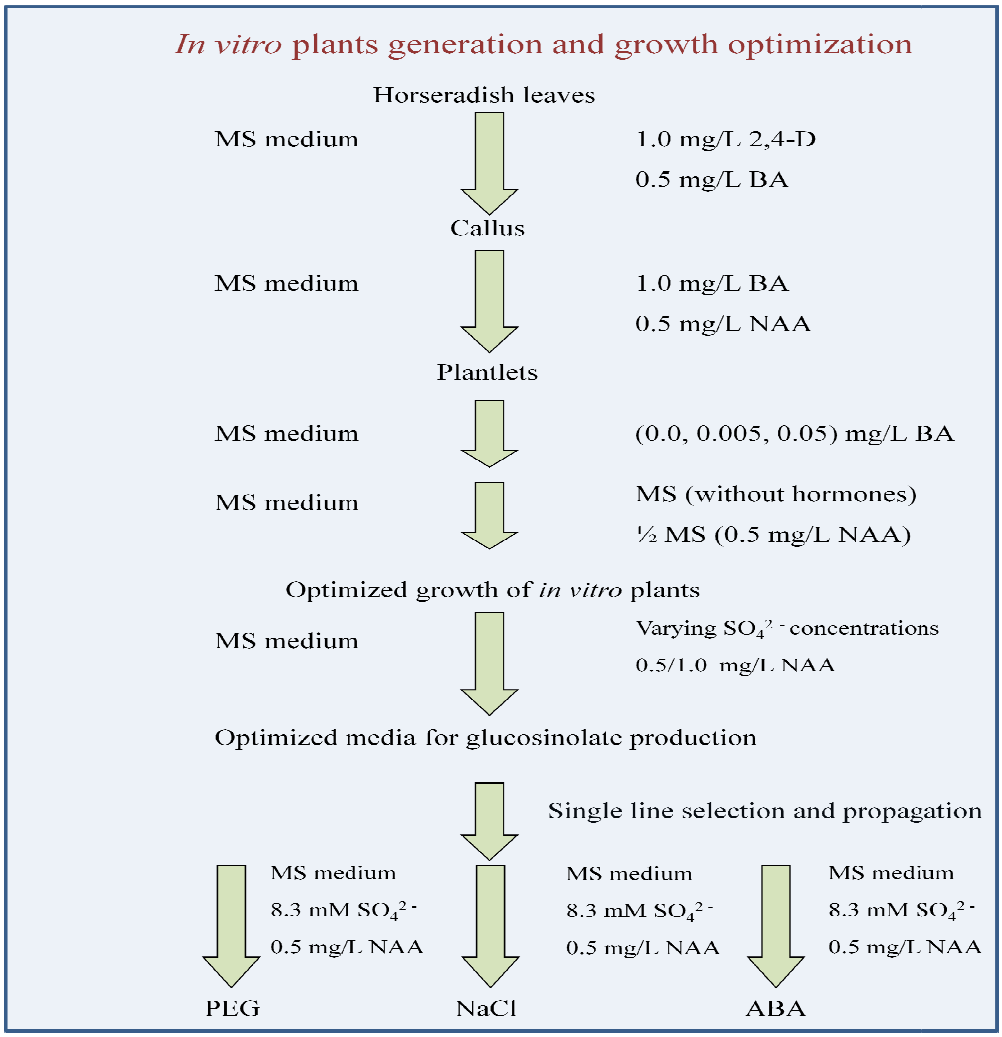


Figure 16: Several steps of *in vitro* plants initiation, growth optimization and plants propagation.

3.2.2. Media preparation

MS media were prepared according to Murashige and Skoog (1962). Different volumes of the stock solutions were used to achieve the required end concentrations of different nutrients. Media and growth regulators used for callus formation, plants regeneration from callus, and growth optimization of regenerated plants are listed in Table 1. Table 2 shows media composition and different treatments applied to investigate the effects of exogenous factors on GS accumulation. Media were supplemented with 3% (w/v) sucrose. Phytohormones and different exogenous factors were added as needed, then pH was adjusted by NaOH and/or HCl to 5.7. Required volume was adjusted with H_2O, and finally 1% agar (0.8% agar in media for callus formation) was added as solidifying agent (Murashige and Skoog, 1962).

Media were filled in 1 L blue capped bottles and autoclaved. 90-100 ml of warm media (40-50 ^oC) were manually dispensed into sterile glass jars and allowed to solidify at room temperature. Jars were left for 48-72 hours before plants were transferred into them (Owen et al., 1991).

Table 2: Media and media supplementations of phytohormones and exogenous factors used to study GS accumulation in horseradish.

Experiment	Medium	Stock solution	Treatments	Phytohormone (NAA)	Other media supplementation
Sulfur concentration	MS (without $MgSO_4^{2-}$)	100 mM $MgSO_4^{2-}$	0.23, 1.7, 8.3 and 21.5 mM SO_4^{2-}	0.5 mg/L NAA	……
NAA	MS	1.0 mg/ml NAA	0.5 / 1.0 mg/L NAA	……..	1.7 mM SO_4^{2-}
PEG	MS	……	0.0, 5.0, 10.0 and 15.0 % (w/v) PEG (MW 20.000)	0.5 mg/L NAA	8.3 mM SO_4^{2-}
NaCl	MS	1.0 M NaCl	0.0, 25.0, 50.0 and 100.0 mM NaCl	0.5 mg/L NAA	8.3 mM SO_4^{2-}
ABA	MS	1.0 mg/ml ABA	0.0, 1.0, 5.0 and 10.0 mg/L ABA	0.5 mg/L NAA	8.3 mM SO_4^{2-}

3.2.2.1. Variation of media

Table 2 shows treatments used in different experiments to investigate the effects of exogenous factors on GS accumulation. First experiments were aimed to optimize the media for GS accumulation of *in vitro* plants. Generally, *in vitro* plants accumulate less GS than

field grown ones. Limited availability of sulfur in the media and limited capacity of the plant to absorb sulfur from the media were proposed as possible reasons (Matallana et al., 2006).

Different sulfur concentrations (0.23, 1.7 (standard), 8.3 and 21.5 mM SO_4^{2-}) effects on GS accumulation were studied. SO_4^{2-} concentrations required were achieved by manipulating $MgSO_4^{2-}$ –the major sulfur source in MS media- concentration. Complete removal of $MgSO_4^{2-}$ resulted in 0.23 mM SO_4^{2-} in the media (minor salts contain SO_4^{2-}). Standard MS medium contains 1.7 mM SO_4^{2-}. 8.3 and 21.5 mM SO_4^{2-} concentrations in the media were achieved by adding $MgSO_4^{2-}$ concentration as needed from 100 mM $MgSO_4^{2-}$ stock. Root system role in sulfur absorption and its effect on GS accumulation were also studied by using typical MS media supplemented with 0.5 mg/L or 1.0 mg/L NAA (Matallana et al., 2006).

For varying the osmotic potential of the media, different polyethylene glycol (PEG) (MW 20.000) amounts were dissolved in MS media supplemented with 8.3 mM SO_4^{2-} and 0.5 mg/L NAA to achieve required end concentrations of PEG; 0.0, 5.0, 10.0 and 15.0% PEG (w/v) (Abdel-raheem et al., 2007; Whalley et al., 1998).

In order to investigate the effect of salt stress, various concentrations of NaCl (0.0, 25.0, 50.0 and 100.0 mM) were added to the media. Similarly, several ABA concentrations (0.0, 1.0, 5.0, and 10.0 mg/L) were adjusted in the media, in order to investigate the role of ABA as abiotic stress signal molecule. In both experiments MS media were supplemented with 8.3 mM SO_4^{2-} and 0.5 mg/L NAA (Keling and Zhujun, 2010; Yuan et al., 2010; Möllers et al., 1999).

In general, stock solutions were used to prepare media for the different treatments. Different volumes of 1 M NaCl stock were used to prepare media of different NaCl treatments. ABA stock (1 mg/ml) solution was prepared by dissolving ABA in 1 N NaOH and required volume was adjusted by H_2O. Different ABA treatments were prepared by adding different stock volumes to the MS media.

Table 3: *In vitro* plants total growth period, plant transfer frequency and sampling schedule for biochemical analysis and dry weight determination of different experiments.

Experiment	Treatment	Total growth period (week)	Transfer within treatment	Frequency (weeks)	Sampling for biochemical analysis	Sampling for dry weight
Callus initiation and plant line regeneration and optimization	Callus initiation	4-5	frequent	4	0	0
	Plant regeneration	7	0	0	0	0
	BA effect	4-5	0	0	0	0
	Nutrient and NAA effect	4-5	0	0	1	0
Exogenous factors effect on GS accumulation	Sulfur effect	6	2	2	3	0
	NAA effect	6	2	2	3	0
	PEG	6	2	2	2	1
	NaCl	6	1	3	1	1
	ABA	6	1	3	1	1

Plants were periodically transferred to new medium as needed in the part of callus formation, plant regeneration and plant growth optimization. In the case of media optimization and and the studies related to the exogenous factors effect on GS accumulation, plants were grown for a total period of six weeks, and transferred to new medium each two weeks at first experiments investigating the effect of SO_4^{2-}, NAA, and PEG. Plants were transferred each three weeks for the rest of experiments (NaCl and ABA). Table 3 shows time periods used to transfer plants in different experiments.

3.2.3. Growth conditions of the *in vitro* plants

Plants were grown at 22 °C with 14/10 hours (light/dark) photoperiod under halogen lights. Illumination intensity was about 100 µE/ s m^2 at the jar surface (Matallana et al., 2006). The plants used in the different experiments (exogenous factors effects on GS accumulation) were propagated on MS media supplemented with 0.5 mg/L NAA and 8.3 mM SO_4^{2-}. Before the *in vitro* plants were used in different experiments, they were allowed to grow for 4-5 weeks.

3.2.4. Sampling

Samples for biochemical analysis were taken at the end of the growth period of all treatments, as well as with each plant transfer to new medium in the case of the trials investigating the effect of sulfur and NAA on GS accumulation. Samples from the PEG experiment were collected after 4 and 6 weeks of treatment (Table 3).

Leaves, stems and roots were excised and directly shock frozen in liquid nitrogen, in order to stop all metabolic activities. Each treatment was conducted with at least 9 *in vitro A.*

rusticana plants grown in 3 different jars (3 plants/ jar). At the end of each experiment (exogenous factors effect on GS accumulation) samples of leaves, stems and roots of two plants from each jar (6 plants for each treatment) were excised and shock frozen in liquid nitrogen. Root samples were briefly rinsed with warm water 30-40 °C in order to remove agar traces, before they were shock frozen.

Different parts (leaves, stems and roots) of each treatment were homogenized separately in liquid nitrogen and lyophilized, then stored at -20 °C. As exception of the general procedure mentioned earlier, samples of different parts from individual plants cultured on medium containing 21.5 mM SO_4^{2-} were collected for GS analysis.

3.3. Dry weight determination

3.3.1. Dry weight determination of roots and leaves of horseradish mature plants

Homogenous samples from roots and leaves were directly weighed to determine the fresh weight, after that they were incubated in an oven at 105 °C until weight was constant. Dried material was allowed to cool to room temperature (RT) in a desiccators and weighed (Breitenstein et al., 2011).

3.3.2. Dry weight determination of horseradish *in vitro* plant parts

Plant materials remaining after sampling for biochemical analysis were used for dry weight (DW) determination. Three plants from each treatment -one plant from three different jars- were transferred to the weighing room while still being in the glass.

Different plant parts of three plants were excised, briefly blotted on tissue paper. Roots were briefly rinsed with warm water (30-40 °C) in order to remove agar traces before being plotted on tissue paper and weighed to determine their fresh weight (FW).

Sample dry weight (DW) was thermogravimetically determined as described by Breitenstein et al. (2011) with slight modifications. Samples of different plant parts were placed in an oven at 105 °C until weight was constant. Dried material was transported in a desiccator and allowed to cool to RT before DW determination.

3.4. Extraction and analysis of glucosinolates

3.4.1. Glucosinolate extraction

GS were extracted according to (Kleinwächter et al., 2008) with some modifications. About 30 mg of dried plant material were weighed in Eppendorf tubes (2 ml). 800 µl MeOH were added, and the tubes were incubated in an ultrasonic water bath at 50 ^{o}C in order to deactivate myrosinase. After 5 minutes 200 µl H_2O were added. In general, the 200 µl water added contained 3 mM sinalbin (4-hydroxybenzyl glucosinolate) as internal standard, resulting in a final concentration of 60 µM. Subsequently 200 µl tetrabutylammonium hydrogensulfate (TBA, 200 mM) was added in order to achieve the required solvent composition for HPLC analysis and incubated for further 5 minutes in ultrasonic water bath at 50 ^{o}C. Following centrifugation at 13.200 rpm for 15 minutes supernatant was collected and gathered with the supernatant of two further extractions (10 minutes each, with MeOH/H_2O (80/20, v/v) solvent), and evaporated in evaporator centrifuge. After MeOH evaporation, Eppendorf tubes were filled to 2 ml with 15% MeOH 85% H_2O, vortexed, and centrifuged at 13.200 rpm for 15 minutes. The supernatant was transferred to 10 ml volumetric flasks and volume was adjusted to 10 ml with H_2O. Finally, samples were filtered by using a disposable filter cartridge (Ø: 25mm/ pore size: 0.2 µm, Macherey-Nagel) and pipetted in 1.5 ml HPLC vials.

3.4.2. Glucosinolates analysis

3.4.2.1. Glucosinolates analysis and quantification

Due to different chemical structures and properties of the GS present in horseradish, ion pair chromatography (IPC) turned out to be the best technique to analyze the different intact GS of horseradish. IPC eliminates the need for tedious extraction and the unpreferable desulfatation step for the production of desulfoglucosinolates. Different to usual reversed phase-HPLC, in IPC the stationary phase (hydrophobic beads) is covered of the ion pair reagent. This allows better retention and analysis of hydrophilic compounds as well as good analysis of aromatic and indolic ones.

Intact GS were analyzed as described by Jen et al. (2001) with some modifications; differing acetonitrile (ACN): H_2O gradients were used as the mobile phase (Table 5), in the presence of 20 mM TBA as ion pair reagent at pH 5.0. In order to clean the column between individual analysis, a strong eluent (60% ACN in water (v/v), 20 mM TBA, pH 5.0) was

briefly used (Table 5). The retention times of sinigrin, sinalbin (internal standard (IS)), and gluconasturtiin were about 7, 10, and 23 minutes, respectively. A standard calibration curve and IS recovery factor were used to quantify the amount of horseradish major GS (sinigrin and gluconasturtiin).

3.4.2.2. Analytical conditions

Devices and analytical conditions used for GS analysis are presented in Table 4.

Table 4: Machines and analysis conditions used for glucosinolate analysis.

Pump	LC Pump, Series 410, Perkin Elmer
Autosampler	Marathon, Spark Holland
Injection volume	50 µL
Detector	LC 90 UV, Spectrophotometric detector; Perkin Elmer
Column, Tempreature	25 x 0,4 cm Nucleosil 120-5-C18, 30 °C
Software	Clarity, version 2.5

3.4.2.3. Solvents compositions and pump analysis program

Intact GS were analyzed as described by Jen et al. (2001) with some modifications. Gradient was prepared by mixing different percentages of acetonitrile (ACN) and 20 mM (TBA) as the ion pair reagent. TBA was prepared in pure water (Ω ≥17.4 µSiemens), and pH adjusted to 5.0 by NaOH. Finally, the TBA solution was filtered through 0.2 µm Wattmann No.1 filter paper (Table 5; Jen et al., 2001).

Table 5: Solvents compositions and pump program used for GS analysis. All solvents were adjusted to pH 5.0, and contain 20 mM tetrabutylammonium hydrogensulfate (TBA) as ion pair reagent.

Time (minutes)	Solvent	Composition ACN/water (v/v)		Flow rate (ml)
0-25	A-B	15-25%	Gradient	1
25-30	B-C	25-60%	Gradient	1
30-35	C-A	60-15%	Gradient	1
35-55	A	15%	Isocratic	1

3.5. GABA extraction and analysis

3.5.1. GABA extraction

GABA was extracted as described by Selmar et al. (2008) with some modifications. About 0.1 to 0.2 g of dried plant material was weighed and transferred to 25 ml volumetric flasks.

About 15-20 ml 4% sulfosalicylic acid (SSA) and 1.25 ml 0.16 mM norvaline as the internal standard were added. Then the flask was shaken vigorously in an ultrasonic water bath at room temperature for 30 minutes. After that, volume of the flasks was adjusted to 25 ml using 4% SSA, shaken well and incubated in the refrigerator at 4 °C overnight. Then, the content of the incubated flasks was mixed and two aliquots were taken from each flask and centrifuged at 10000 rpm for 10 minutes at 4 °C. Supernatant was filtered by HPLC certified disposable syringe filter (Ø: 25 mm/pore size: 0.2 µm, Macherey-Nagel). Finally, 750 µl of the extract were pipetted into HPLC vials. GABA amount was determined according to standard calibration curve and internal standard recovery factor.

3.5.2. GABA analysis

3.5.2.1. Machine specifications and data analysis program

Table 6 shows devices, analytical conditions and software used for GABA analysis.

Table 6: Different parts specifications, analytical conditions and software used for GABA analysis.

Pump	Techlab
Autosampler	Midas, Spark Holland
Injection volume	100µl
Detector	Shimadzu RF 551-Fluorescence detector
Excitation	334 nm
Emission	425 nm
Column	20x0.4 cm, Nucleosil, 100-5-C18
Software	Clarity, version 2.5
Temp	35 °C

3.5.2.2. Autosampler program for amino acids derivatization by *o*-phthalaldehyde

Autosampler automated program for amino acids derivitization by *o*-phthalaldehyde (OPA), and OPA reagent composition are presented in Table 7. Injection is performed two minutes after OPA addition to the sample (Selmar et al., 2008).

Table 7: Detailed program of amino acids derivitization by OPA automatically performed by autosampler, and OPA reagent composition.

Step Nr	Action	Amount	Reagent
1	Add	750 µl	Sample
2	Add	280 µl	1 N NaOH
3	mix	999 µl	……
4	Add	375 µl	OPA reagent →
5	Mix	999 µl	……
6	Injection	100 µl	……

50 mg OPA
1.2 ml MeOH
50 µl mercaptoethanol
11.2 ml 0.2 M boric acid buffer

3.5.2.3. Solvents compositions and pump program for GABA analysis

GABA analysis was conducted as described by Selmar et al. (2008) with some modifications; sodium acetate (NH_4Ac) buffer (50 mM, pH 6.2) was prepared in H_2O ($\Omega \geq 17.4$ μSiemens). Different volumes of NH_4Ac and organic solvents were used to prepare different solutions for GABA analysis. Compositions of different solutions used for GABA analysis are presented in Table 8, while Table 9 presents the GABA analysis program of the pump.

Table 8: Composition of different solvents used for GABA analysis.

Solution		
A	**B**	**C**
1800 ml 50 mM sodium acetate buffer, pH 6.2	200 ml 50 mM sodium acetate buffer, pH 6.2	……
100 ml ACN	400 ml ACN	…….
100 ml MeOH	400 ml MeOH	800 ml MeOH
40 ml tetrahydrofuran (THF)	……..……..	……..
……..	…….	200 ml H_2O

Table 9: Solvent program used for GABA analysis.

Time (min)	Solutions percentages			Flow rate (ml)
	A	**B**	**C**	
0	100	0	0	1
12	100	0	0	1
27	90	10	0	1
39	74	26	0	1
44	74	26	0	1
48	60	40	0	1
63	50	50	0	1
68	0	0	100	0.8
71	0	0	100	0.8
75	100	0	0	1
78	100	0	0	1

3.6. Myrosinase extraction and characterization

3.6.1. Myrosinase extraction

Crude extract of soluble myrosinase was prepared as described by (Kleinwächter and Selmar, 2004) with some modifications; 100 mg of freeze dried plant materials were homogenized with an ultra turrax in 10 ml 25 mM Sørensen buffer at pH 5.7 for 30 seconds. Then, the homogenate was filtered through a cheese cloth. In order to remove endogenous glucose and ascorbic acid, aliquots of the extracts were cleaned up by PD-10 desalting columns

(Pharmacia). For this, 2 ml of the extracts were centrifuged for 1 hour (13.200 rpm, 4 °C). Then, 2 ml of the supernatant were placed on the PD-10 desalting columns, followed by 0.5 ml 25 mM Sørensen buffer (pH 5.7). Crude extract was eluted by 2.5 ml Sørensen buffer and stored at -20 °C until needed.

3.6.2. Myrosinase characterization

Horseradish myrosinase was characterized with the respect to the effect of basic parameters on enzyme activity. Activity was measured as the amount of glucose liberated, when the enzyme was incubated with sinigrin in the presence of 50 µl (1 mM) arabinose as internal standard. The effects of the substrate concentration (sinigrin), ascorbic acid concentration, pH and temperature on the enzyme activity were investigated (as described in Table 10).

Table 10: Parameters varied and reaction conditions used for myrosinase basic characterization.

Parameter investigated	**Reaction conditions**	
Ascorbic acid concentration (0-10 mM)	Sinigrin	1 mM
	Temp	30 °C
	Time	20 min
	pH	25 mM, 5.7 Sorensen buffer
Sinigrin concentration (0-5 mM)	Ascorbic acid	2 mM
	Temp	30 °C
	Time	20 min
	pH	25 mM, 5.7 Sorensen buffer
pH (3-8) **(pH 3-4.5, McIlvaine buffer)** **(pH 5-8, Sørensen buffer, 25 mM)**	Sinigrin	2 mM
	Ascorbic acid	2 mM
	Temp	30 °C
	Time	20 min
Temperature °C (4, 20, 30, 40, 47, 50, 60)	Sinigrin	2mM
	Ascorbic acid	2 mM
	Time	20 min
	pH	25 mM, 5.7 Sørensen buffer

Enzyme reactions were carried out as described by Kleinwächter and Selmar (2004) with some modifications; reactions were started by the addition of 10 µl enzyme[1] to the reaction mix. After incubation for 20 minutes, tubes were shock frozen in liquid nitrogen to terminate the reaction, and the reaction mixture was lyophilized over night. In order to extract the lyophilized powder, 1 ml pure MeOH was added, then the tubes were briefly vortexed, and incubated in an ultrasonic water bath for 10 minutes at room temperature. After centrifugation (13.200 rpm, 10 minutes), the supernatant was transferred to another tube, and

[1] Enzyme concentration was diluted after the first experiment concerning ascorbic acid effect for optimal activity measurement.

pellet underwent further extraction with 0.5 ml pure MeOH as described previously. Then, the supernatants were combined, and the MeOH was removed in an evaporator centrifuge. Residues were dissolved by addition of 1 ml ultra pure water and filtrated using HPLC certified disposable syringe filters (Ø: 25 mm/pore size: 0.2 µm, Macherey-Nagel). For myrosinase activity measurements from varying treatments in the rain shelter, protein concentration was quantified using a Bradford assay kit (Roti-Quant, Carl-Roth. Germany) and protein concentrations were adjusted to 0.1 µg/ml with Sørensen buffer (25 mM, pH 5.7). Standard conditions were used for the enzyme reactions: 2 mM Ascorbic acid, 2 mM sinigrin, pH 5.5 and 40 °C.

3.6.3. Myrosinase activity determination by means of glucose liberated.

3.6.3.1. Devices and analysis conditions

Machines, machines parts and analysis conditions used for soluble sugars determination is presented in Table 11.

Table 11: Devices and analysis conditions used for glucose determination.

Pump	Dionex, GS 50 gradient pump with borat trap
Autosampler	Dionex, AS 50 Autosampler with AS 50 thermal compartment
Injection volume	20 µl
Detector	Dionex, ED 50, Electrochemical detector with disposable golden electrode.
Detection	Pulsed amperometric
Column	Dionex, carboPac PA20 column
Pre-column	Amino trap
Software	Chromeleon, version 6.40
Tempreature	30 °C
Flow rate	0.5 ml/ min

3.6.3.2. Pump analysis program

Soluble sugars were determined by high performance anion exchange chromatography pulsed amperometric detection (HPAEC-PAD) as described by (Knopp, 2005; Selmar et al., 2008). Different NaOH concentrations were used as mobile phase (Table 12). The amounts of glucose liberated were calculated in relation to a glucose calibration curve and the recovery rate was determined by means of of externally added arabinose as internal standard (ISS). Only ultra clean H_2O (Ω =18.2 µSiemens) was used for the preparation of the NaOH stock solutions and the glucose analysis.

Table 12: NaOH concentrations used for soluble sugar analysis.

Time (min)	NaOH [mM]
0	17
9.5	12.5
9.51	28
17.5	28
17.51	108
29.01	108

3.7. Statistical analysis and calculations

3.7.1. Statistical analysis

Data obtained were statically analyzed by SPSS v 8.0 according to student T test at 0.05 degree of confidence.

3.7.2. Glucosinolate and GABA concentrations on whole plant basis

GS and GABA concentrations in different organs on dry matter basis were directly determined. Their concentrations on fresh weight basis were determined in relation to the water content of the different organs. In order to calculate GS and GABA contents in the whole plant on dry and fresh weight basis, individual organs contribution to the total plant weight and their GS and GABA concentrations on dry or fresh weight basis were considered.

3.8. Repetition of field experiment

In order to confirm the results obtained from the field experiment in 2010, a corresponding trial was repeated in 2011. Similar to the previous experiment, soil designs were created (furrows and ridges) to cause variations in the water supply for horseradish plants from the Badisch and the Kröner variety (30 plants from each variety; see also Figure 11). However, for more simplicity in data analysis, different to the previous experiment, salinity effects were not investigated. Plants preparation, cultivation, harvest and field maintenance were conducted as described previously (chapter 3.1.). For sampling, homogenous samples were taken from 15 randomly chosen plants. These samples were used for dry weight determination and biochemical analysis.

4. Results and Discussion*

This work is aimed to elucidate the effects of exogenous factors on GS accumulation in horseradish plants. Among these factors, the most widely spreaded abiotic stresses, i.e., drought and salinity stress are considered. Moreover, direct effects of important signaling molecules, such as ABA and SA, on GS accumulation have been investigated. These molecules are part of plant signaling mechanisms, and thus regulate plant responses to exogenous factors. Accordingly, this study will help elucidating the complex mechanisms of plant responses to abiotic factors. In particular, effects on plants secondary metabolism, especially on GS accumulation in horseradish plants will be investigated.

In order to exemplarily investigate the complex interaction between plant abiotic stress, usually caused by extreme abiotic factors, and its effects on GS accumulation, experiments were conducted on *in vitro* horseradish plants as well as on intact plants cultivated under rain shelter and in the field. Closed culture systems will allow investigating the effect of single stresses on horseradish plants under controlled environments, i.e., under *in vitro* conditions. Whereas plants grown in the field are known to be challenged with multiple stresses. However, the applicability of abiotic factors to improve the quality of horseradish tubers was investigated in plants grown in rain shelter and in the field.

An experimental prerequisite for our investigations was to use homogenous horseradish plants in the different experimental systems. Due to the common agricultural practice to propagate horseradish vegetatively, varieties with homogenous mature plants are easily obtainable. On the other hand, homogenous horseradish *in vitro* plants had to be regenerated. Moreover, the growth of these plants had to be optimized before using them in the different experiments.

* Due to multidisciplinary work carried out in this thesis, and for clear presentation of results and discussion, corresponding discussion is compiled at the end of each section after complete results have been presented.

4.1. Effects of abiotic exogenous factors on glucosinolate accumulation in horseradish *in vitro* plants

In the following chapter, the experiments elucidating the impact of exogenous abiotic factors on GS accumulation in horseradish *in vitro* plants are presented. For this investigation, *in vitro* horseradish plants had to be regenerated and their growth had to be optimized. Then, a sufficient number of homogenous *in vitro* plants should be regenerated by asexual propagation.

4.1.1. Line initiation of horseradish *in vitro* plants

In this chapter the initiation of *in vitro* horseradish plant lines from explants is presented. *In vitro* plant line initiation is the first step toward mass production of *in vitro* plants. Then, these plants will be used to investigate the effects of abiotic exogenous factors on GS accumulation.

Horseradish generally is propagated vegetatively, since its seeds reveal a low viability (Courter and Rhodes, 1969). Therefore, many procedures were reported for horseradish *in vitro* plants regeneration, e.g., using hairy roots, direct shoot regeneration and shoot regeneration from callus (Araki et al., 1995; Pawelczak et al., 2006; Uozumi et al., 1994; Nakashimada et al., 1996).

Unfortunately, first experiments on direct plant regeneration from leaf explants failed. Cultured explants did not regenerate *in vitro* plants directly. However, callus was formed from explants cultured on MS media supplemented with 1.0 mg/ L 2,4-D and 0.5 mg/ L BA. Then, the produced callus was used to regenerate plants by culturing it on MS media supplemented with 0.5 mg/ L NAA and 0.1 mg/ L BA.

The direct plant regeneration from leaves might be unsuccessful, due to age of the explants. Araki et al. (1995) and Pawelczak et al. (2006) reported that the age and collection season of the explants affected plantlet regeneration. Moreover, they concluded that media composition should be adapted for particular horseradish types (Pawelczak et al., 2006). It was reported that leaves explants tend to form callus, when cultured on medium containing more than 0.1 µM 2,4-D (Araki et al., 1995; Yuliadi, 2008).

For the performed investigations different lines of plants were regenerated from callus. These lines were separated into different growing jars, labeled and asexually propagated, to ensure plant line homogeneity.

4.1.2. Growth optimization of horseradish *in vitro* plants

Plants regenerated from callus developed no or poor root system. Therefore their growth and development had to be optimized. In order to enhance root growth, plants were transferred to MS media supplemented with 0.5 mg/L NAA and varying concentrations of BA (0.0, 0.005 and 0.05 mg/L). All plants developed root systems, but the growth medium lacking BA was the best medium for root formation. The roots formed from plants grown on media supplemented with BA were short, thick and bulky when compared to those formed on media lacking BA supplement. Moreover, plants cultured on media supplemented with low BA (0.005 mg/L) formed better roots systems compared to the plants cultured on media supplemented with the higher BA (0.05 mg/L) concentration. Comparison of plants cultured on MS media supplemented with 0.5 mg/L NAA and 0.0, 0.005 and 0.05 mg/L BA respectively, is presented in Figure 17. From the habitus displayed in Figure 17, it can be deduced that plants grown on higher BA concentration (0.05 mg/L) are more vigorous compared to plants grown on media supplemented with low BA concentration (0.005 mg/L) or lacking BA supplement. Yet, plants grown on media containing high BA supplements tend to form callus more often than those grown without BA.

Figure 17: *In vitro* horseradish plants cultured on MS media supplemented with 0.5 mg /L NAA and 0.0, 0.005 and 0.05 mg/L BA after 7 weeks of cultivation.

The growth of the *in vitro* plant was further optimized by sub-culturing the regenerated plants on MS media lacking any growth regulators or ½ MS media (MS media containing half amount of salts) supplemented with 0.5 mg /L NAA. Plants grown on ½ MS supplemented

with 0.5 mg /L NAA showed better growth and root system formation (Figure 18, B & D) compared to plants cultured on MS media lacking any hormone supplementations (figure 18, A & C).

These findings are in agreement with previous reports concerning BA effects on horseradish plants. Pawelczak et al. (2006) concluded that in the case of shoot direct regeneration from root explants, BA concentrations higher than 0.1 mg/ L caused leaf deformation and inhibition of rooting (Pawelczak et al., 2006). Moreover, plants cultured on high BA containing medium showed more vigorous growth compared to plants cultured on media containing lower or no BA supplements. This might be the reason of the previously reported higher weight of plants cultured on growth medium with higher BA concentrations (Pawelczak et al., 2006).

Moreover, these authors also found that lacking of hormone supplementations in the culture medium only affected shoots weight of regenerated plants (Pawelczak et al., 2006).

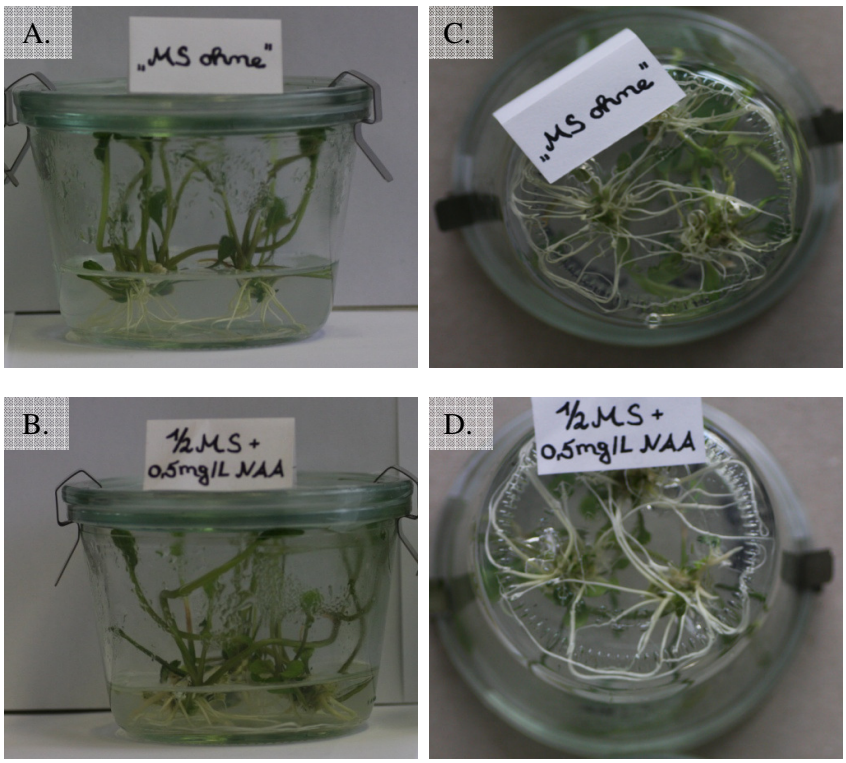


Figure 18: Growth optimization of horseradish *in vitro* plants. A and B present a comparison of the habitus of *in vitro* plants cultured on MS media without hormone supplementation (A), or on ½ MS media supplemented with 0.5 mg/L NAA (B). C and D focus on the root system of plants cultured on MS media lacking NAA (C) and ½ MS media supplemented with 0.5 mg/L NAA (D). Photos were taken 2 weeks after plant transfer to media.

According to the results presented above, for the experiments carried out for this thesis, MS media supplemented with 0.5 mg/L NAA for optimum rooting of cultured plants, and lacking 2,4-D or BA supplementation seem to be appropriate. For further propagation, ½ MS media supplemented with 0.5 mg/L NAA were found to be more suitable than MS media lacking NAA supplementation.

Horseradish *in vitro* plants regeneration and their growth optimization were successful. In the next step *in vitro* plants were mass propagated to generate a large number of plants, which were used in the required experiments. However, before the plants can be used in further experiments, a reliable method for GS determination in small plant samples had to be developed.

4.1.3 Method development for the extraction and analysis of intact glucosinolates in different parts of horseradish *in vitro* and mature plants

Numerous methods for the quantification of GS in plant material are available (Olsen and Sorensen, 1981), however, no sound method is reported for the analysis of GS in very small amounts of plant material; such as parts of *in vitro* plants. Accordingly, a reliable method for the determination of the small amounts of GS present in *in vitro* plants had to be established in order to analyze the different parts of the plants with different developmental stages. Apart from the challenge to determine very low amounts, the corresponding plant parts may contain various metabolites that might interfere with GS determination.

As result of various analytical approaches, it turned out that the following method accomplished the requirements best: Intact GS were extracted from natural samples and then analyzed using ion-pair high performance liquid chromatography (IPC). IPC is a technique that allows the analysis of intact GS, eliminating the need for GS deslufatation.

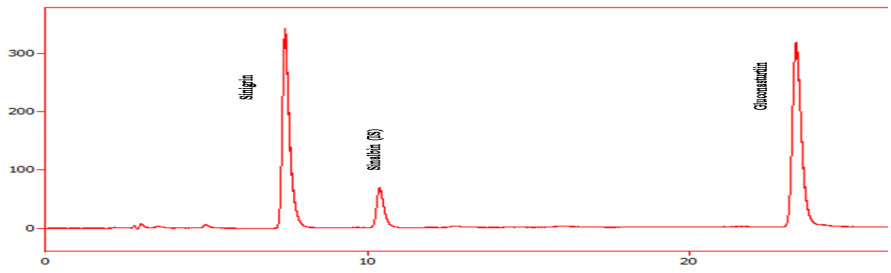


Figure 19: Chromatogram of glucosinolate standards. SI and GN were used as reference materials for GS quantification, sinalbin (SB) was used as internal standard (IS).

Intact GS in natural samples were identified and quantified by comparison to standards, i.e., sinigrin (SI) and gluconasturtiin (GN). The GS in natural samples had frequently been identified by direct addition of these references. Sinalbin (SB) (4-hydroxybenzyl glucosinolates), which is absent in horseradish, was used as the internal standard. A corresponding chromatogram is displayed in Figure 19.

Discussion

As interest in determining GS concentrations in different plants is relatively old, a considerable number of methods has been applied (Olsen and Sorensen, 1981). Among them the quantification of desulfoglucosinolates, obtained by desulfatization of GS, by reversed phase high performance liquid chromatography (RP-HPLC) is the most practiced one. However, the tedious extraction steps at high temperatures and the incubation with desulfatase make it less convenient. Alternatively, several reports were published, which enable intact GS extraction and analysis without the need for the desulfatation step. Here a method was developed for intact GS extraction and analysis. This method depends on previously described procedures for intact GS extraction and analysis with some modifications (Kleinwächter et al., 2008; Jen et al., 2001). Moreover, SB was chosen as the internal standard, because it does not occur in horseradish (Fahey et al., 2001).

4.1.4. Determination of glucosinolate concentrations in mature and *in vitro* horseradish plant parts

In order to verify and approve IPC capacity for reliable determination of GS in different parts, mature plants (roots and leaves), and (leaves and stems) of *in vitro* plants as well as in callus derived from these plants were analyzed for their GS concentrations.

Root samples of mature plants revealed the highest GS concentration (≈ 235 µmole/g d.w; Figure 20), and leaves exhibited lower GS concentrations (≈ 100 µmole/g d.w.). In contrast to these concentrations in intact plants, *in vitro* plants reveal much lower GS concentrations (≈ 100 versus 21 µmole/g d.w.). Callus samples exhibit only traces of GS. In almost all samples of differentiated tissues and organs, sinigrin (SI) was much higher concentrated than gluconasturtiin (GN). Only in callus samples GN was higher than that of SI (Figure 20).

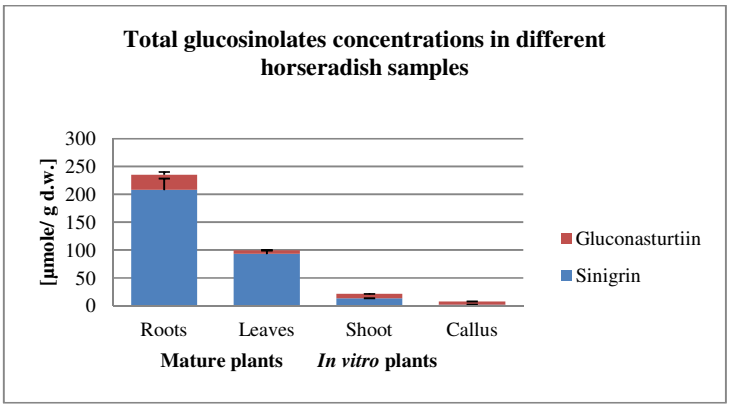


Figure 20: Total glucosinolate concentrations of main roots, and leaves of mature plants, and shoots (leaves and stems) of *in vitro* plants as well as of callus. Data represent three different extractions. Error bars resemble standard deviation (StD).

SI and GN represent the major GS in horseradish, other GS only could be detected in traces. These results are in agreement with previous reports (Redovnikovic et al., 2008b; Li and Kushad, 2004). Therefore, for further investigations in this thesis, only SI and GN had been analyzed and accounted to present the total glucosinolate (total GS) concentrations in corresponding samples.

The high total GS concentration (≈ 235 µmole/g d.w.) found in roots of mature plants is in accordance with the literature. Due to its nature as storage organ, horseradish roots could contain concentrations of GS up to nearly 300 µmole/g d.w. (Li and Kushad, 2004). Low GS concentrations in callus have been previously been reported. This finding was expected, since callus tissues lack specialized organs. Accordingly, biosynthesis and accumulation of GS are strongly diminished (Alnsour et al., 2012; Redovnikovic et al., 2008b). Also the lower concentrations of *in vitro* plants have been reported earlier (Li and Kushad, 2004; Redovnikovic et al., 2008b). As *in vitro* plants reveal specialized and differentiated tissues as like leaves from intact mature plants, huge differences between leaves from mature plants (≈ 100 µmole/g d.w.) and *in vitro* plant shoots (≈ 21 µmole/g d.w.) are astonishing. The question arises, what causes these high differences in GS accumulation.

Apart from putative effects of developmental stages, literature indicated that this difference may mainly be due to a sulfur supply limitation of the growth medium. This might be caused either by too low sulfur concentrations in the growth medium, or by a too low capacity of the *in vitro* plants root system to take up the required amounts of sulfur for GS biosynthesis (Matallana et al., 2006; Alnsour et al., 2012).

In order to avoid any sulfur depending limitations of GS accumulation that might interfere with stress related impacts on GS metabolism, this issue had to be clarified. Accordingly, the effects of both hypotheses on GS accumulation had been investigated. However, before principally investigating the effect of sulfur supply and uptake on GS accumulation, The effect of somaclonal variation on GS accumulation should be elucidated in order to establish sound experimental conditions for further experiments.

4.1.4.1. Effect of somaclonal variation on the glucosinolate concentration of horseradish *in vitro* plants

In order to estimate the range of somaclonal variation, different *in vitro* plant lines regenerated from callus were analyzed. All different plant lines (IV1-IV6) were grown under identical conditions on ½ MS media supplemented with 0.5 mg/L NAA, and GS concentrations were determined. In order to determine the standard deviation, numerous individual plants belonging to the same plant line were also investigated.

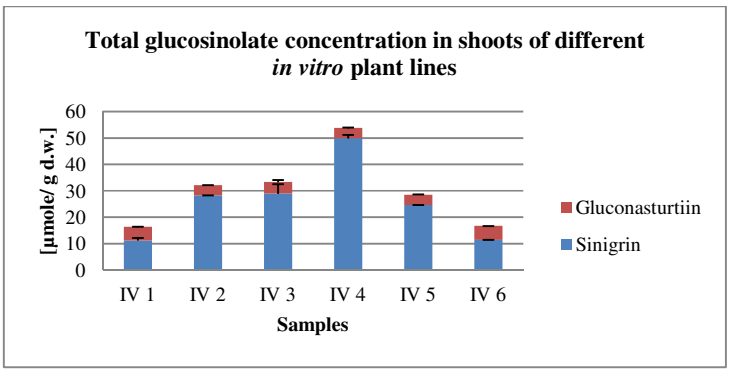


Figure 21: Total GS concentrations in shoots of different *in vitro* plants line (IV1-IV6) regenerated from callus. Error bars resemble StD.

Results presented in Figure 21 show clearly that different plant lines contain different total GS concentrations, varying from about 16 to 54 µmole/g d.w. Interestingly, the noticed differences are mainly due to differences in SI concentrations. GN concentrations are generally low and do not vary markedly among various plant lines. Consequently, GN has low impact on the observed differences.

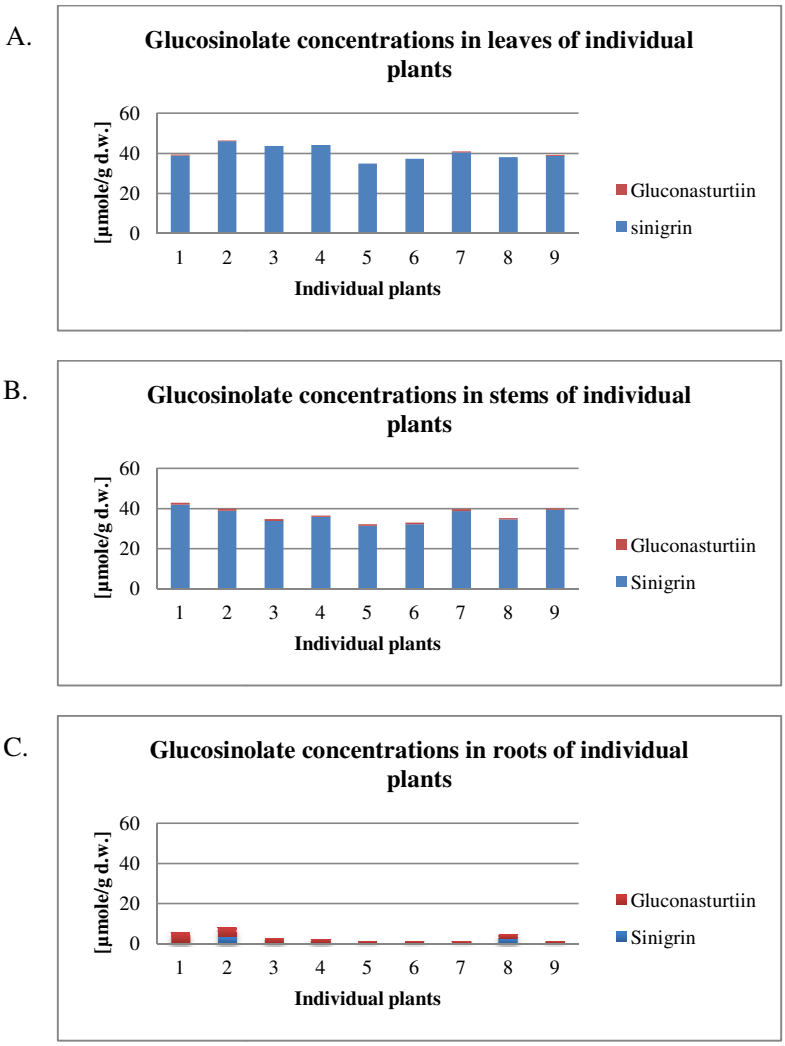


Figure 22: SI and GN concentrations in leaves (A), stems (B) and roots (C) of nine individual plants of one *in vitro* line.

To investigate if the different manifestation is due to the organ specific distribution of SI and GN, different organs of *in vitro* plants had to be analyzed separately. On a first glance, different *in vitro* plant organs reveal different pattern of SI and GN accumulation (Figure 22). Leaves accumulated almost no GN, while in stems traces of GN could be detected. In contrast, in roots SI is absent in most samples. Trace concentrations of GN in stems and trace concentrations of SI in roots might be caused by imperfect cutting of the organs. Especially separating stems from roots, might cause "cross-contamination" with compounds present in the different organs (Figure 22). Apart from the possibility of differential *in situ* biosynthesis of specific GS in various organs, the observed pattern of SI and GN accumulation in specific organs might be caused by translocation from one organ to another for certain putative functions.

As expected, and previously reported, the *in vitro* plants regenerated from callus show a considerable degree of somaclonal variation (Rostiana et al., 1999). Therefore, one line was chosen and propagated vegetatively to be used later in the different experiments. This line was designated as line 1.

After generating a suitable number of homogenous *in vitro* plants, the effect of the sulfur concentration in the medium and the effect of the root system on sulfur uptake from the medium and the subsequent effects on GS accumulation were investigated.

4.1.5. Effect of the sulfur supply on the glucosinolate accumulation in horseradish *in vitro* plants

Sulfur was found to affect GS accumulation markedly, and as the *in vitro* plants accumulate much lower GS concentrations as compared to plants grown in field, it could be assumed that insufficient sulfur uptake is the reason for the low GS accumulation in *in vitro* plants. Corresponding findings were reported for nasturtium *in vitro* plants (Matallana et al., 2006). These authors demonstrated that the sulfur supplement in the culture medium enhances the GS concentration in *in vitro* plants. Accordingly, the effects of the sulfur concentration on GS accumulation in horseradish *in vitro* plants were investigated.

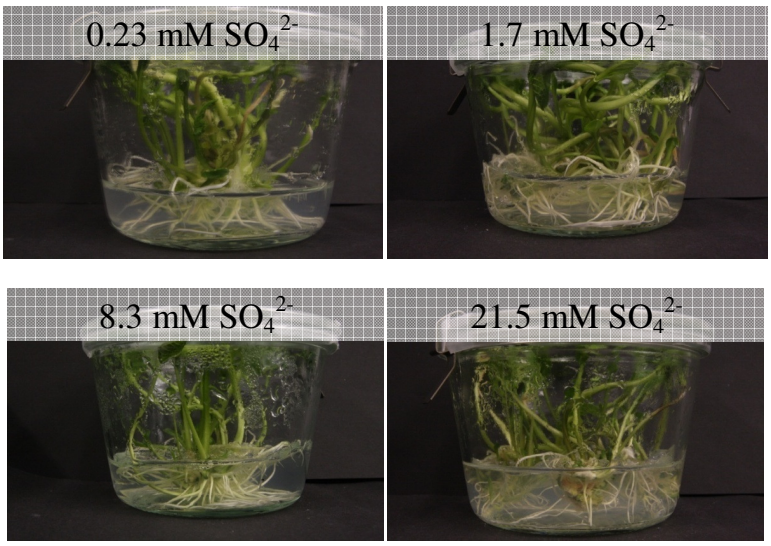
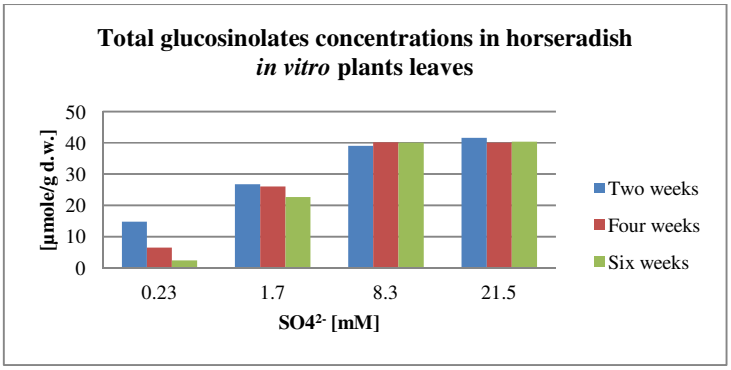


Figure 23: Horseradish *in vitro* plants after 4 weeks of culture on MS media supplemented with 0.5 mg/L NAA and 0.23, 1.7 (standard), 8.3 and 21.5 mM SO_4^{2-}.

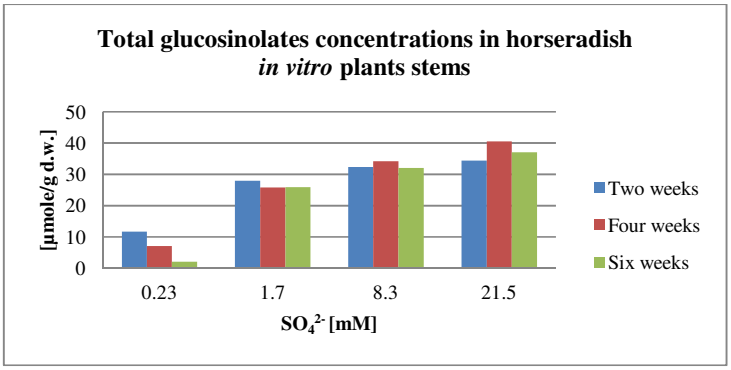
In Figure 23 *in vitro* horseradish plants grown on MS media supplemented with varying amounts of sulfur (0.23, 1.7 (standard), 8.3 and 21.5 mM SO_4^{2-}) are displayed. Plants grown

on standard MS-medium (1.7 mM SO_4^{2-}) show the best growth and the best root system as compared to the other treatments. On the other hand, typical characteristics of plants cultured on high sulfur media, such as retardation in root system formation and abnormal roots proliferation of the hypocotyls, were only observed in plants cultured in medium containing 21.5 mM SO_4^{2-}.

A.



B.



C.

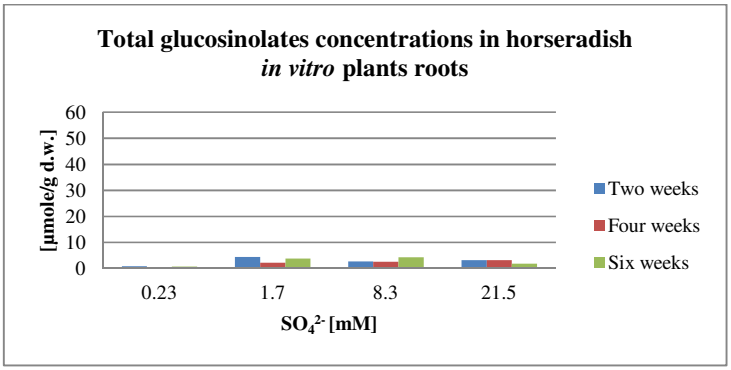


Figure 24: Total GS concentrations in leaves (A), stems (B) and roots (C) of horseradish *in vitro* plants over a growth period of six weeks. Presented data correspond to the average of two independent extractions, except for plants cultured on MS media supplemented with 21.5 mM SO_4^{2-} after six weeks of culture. In this case, data are average of nine individual extractions and analysis of different plant parts (leaves, stems and roots) of nine different plants. Detailed data of SI and GN concentrations as well as the standard deviation values are available in the appendices (Table A2 & Figure A1).

To obtain a comprehensive picture of the effects how sulfur impacts on total GS concentrations of leaves, stems and roots, samples had been taken each two weeks for an overall growing period of six weeks; the corresponding data are presented Figure 24 (A-C). In Figure 25 the same data are displayed. However, more emphasis was given to sulfur effects on total GS concentrations over the time in horseradish *in vitro*. For total GS concentrations at the beginning of the experiment (0 weeks), total GS average from organs of plants cultured on standard media over 6 weeks were used.

Data presented for total GS in leaves and stems (Figure 24 (A & B) and Figure 25) clearly show a strong dependence of sulfur supplementation and total GS biosynthesis in these photosynthetic organs, which possess the capacity to biosynthesize GS. Sulfur supplementation markedly increased total GS accumulation in these organs.

Changes in total glucosinolates concentrations in various *in vitro* plants organs in response to sulfur treatments over time

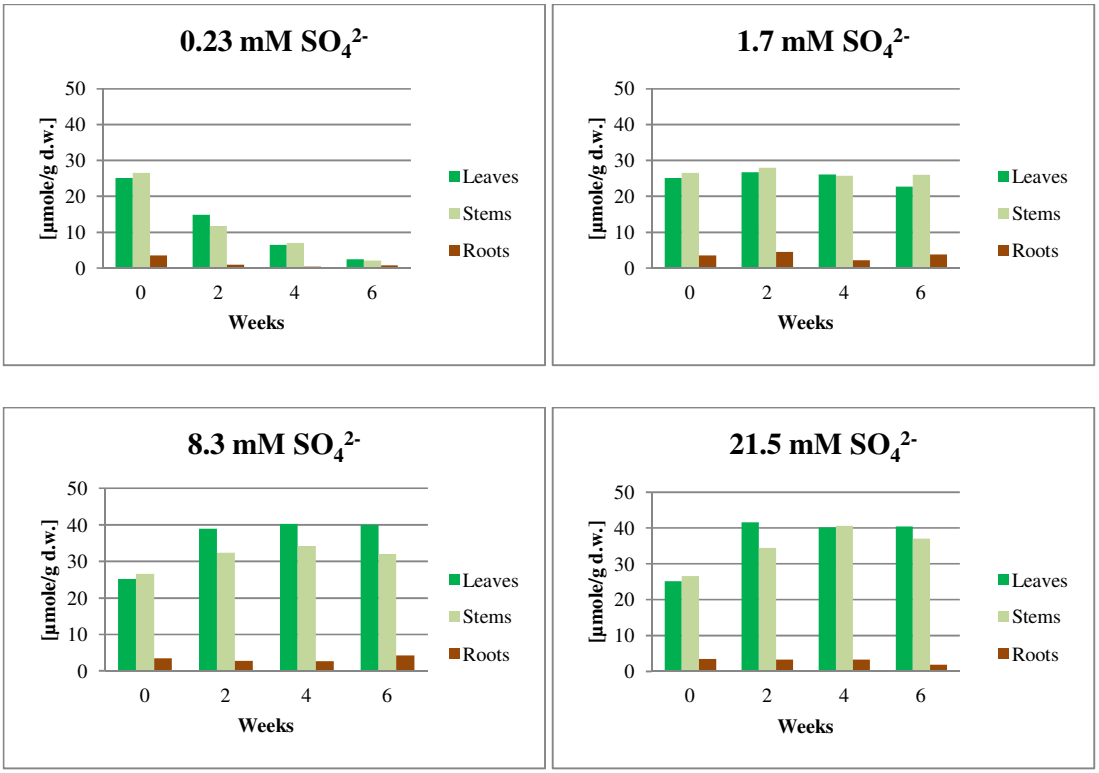


Figure 25: Changes of total GS concentrations over time in leaves, stems and roots of horseradish *in vitro* plants cultured on MS media supplemented with varying concentrations of sulfate. Total GS average from organs of plants cultured on standard (1.7 mM SO_4^{2-}) media over 6 weeks were used for initial total GS concentrations (0 weeks).

Total GS concentrations decreased gradually over time in *in vitro* plants leaves and stems cultured on sulfur deficient MS media, while they remained constant in plants cultured on standard (1.7 mM SO_4^{2-}) MS media (Figure 24 (A & B) and Figure 25). The enhancement of SO_4^{2-} concentrations from 8.3 to 21.5 mM SO_4^{2-} resulted in slight increase in total GS concentrations (Figure 24). Moreover, it caused negative effects on *in vitro* plants growth, such as reduced root formation and hypocotyls abnormal proliferation, showed in Figure 23.

Among all organs roots had the lowest total GS concentrations; total GS concentrations did not exceed 5 µmole/g d.w. (Figure 24 (C) and Figure 25). However, in roots, total GS concentrations decreased in response to sulfur limitations, but they did not increase in case of sulfur supplement (Figure 24 (C) and Figure 25).

The time based data rearrangement, clearly displays three different patterns in response to sulfur supplements (Figure 25). Firstly, due to sulfur limitation GS concentrations decreased gradually over time. Only traces of GS were found after six weeks of horseradish plants culture on sulfur deficient media. Secondly, in plants cultured on standard media, almost no change was observed over the culture period of six weeks. Thirdly, in response to enhanced sulfur supplementations GS concentrations increased significantly.

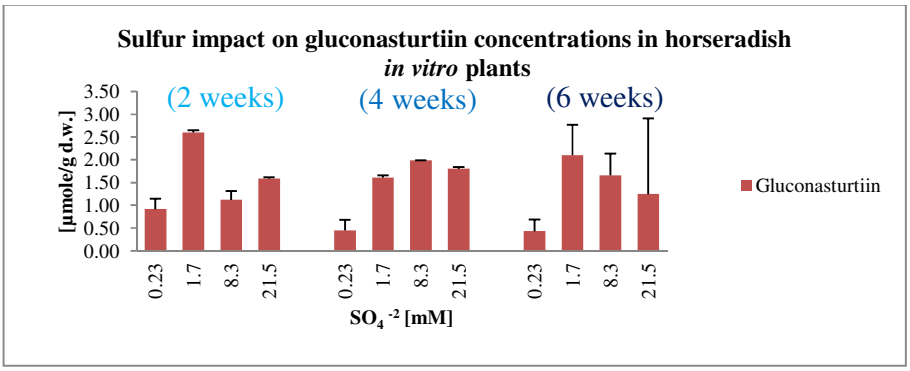


Figure 26: GN concentrations in roots of *in vitro* horseradish plants cultured on media supplemented with various sulfur concentrations over six weeks culture period.

Similar observations had been made by Matallana et al. (2006): Negative effects on the growth behavior of plants cultured on media with elevated sulfur concentrations were also observed in *T. majus in vitro* plants subjected to high sulfur supplementations. Moreover, the results obtained for GS concentrations in leaves and stems of horseradish *in vitro* plants are in agreement with data presented in the literature (Matallana et al.,2006). For review see (Falk et al., 2007; Selmar and Kleinwächter, 2013).

As already mentioned, plants suffering from sulfur starvation accumulated far lower concentrations of SI and GN, and sulfur supplementations resulted in enhanced concentrations of SI. In contrast, the enhancement of sulfur supplementations has only minor effects on the GN concentrations. For simplicity, these coherences are depicted in Figure 26, where the GN concentrations in the roots (plant organ with highest GN concentrations) of horseradish *in vitro* plants cultured on MS media with various sulfur supplementations are displayed. Complete details of sulfur impacts on SI and GN concentrations in various organs of horseradish *in vitro* plants are presented in the appendix (Figure A1).

In contrast to the massive storage roots of soil grown horseradish plants, in *in vitro* plants roots do not represent a storage organ. They provide the plant with water and nutrients. Accordingly, they are thought to contain lower GS concentrations. GS translocation from source organs (leaves and stem) to roots obviously is limited in contrast to the storage "sink" of the tap root of the mature plants, which are known to accumulate high GS concentrations (Li and Kushad, 2004).

The GS decrease in sulfur deficient media may have two possible reasons; this decrease might be due to a dilution effect of GS concentration as consequence of plants vegetative growth while no GS are synthesized. Alternatively, this effect could be caused by a remobilization of GS *in vivo* in order to mobilize the sulfur of the GS for the sulfur supply of primary metabolism. Thus, mobilized elements, such as sulfur, can be utilized in other cellular metabolism processes. GS mobilization *in vivo* was already proposed by Chen and Andreasson (2001). Unfortunately, up to now no sound data on this issue are available. To decide whether or not in the horseradish *in vitro* plants GS have been remobilized during sulfur starvation, the overall content of GS in all plants at the beginning of the experiment and at the end had to be calculated. For this, however, the biomass of all *in vitro* plants must be known. Unfortunately, the experimental setup was not adequate to address this subject properly, since the estimation of biomass had to be omitted in order to keep the number of plants in an appropriate range. However, sulfur starvation could be a valuable tool to address GS mobilization *in vivo* in future research, *in vitro* plants should be transformed to sulfur deficient media and the time course of the total GS have to be examined.

The finding that the pattern of GS accumulation in plants cultured on standard MS media remains almost constant was expected, since no changes in growth conditions had been applied.

As consequence of sulfur enhancement, GS accumulated to higher levels compared to control plants. This increase indicates that higher levels of GS are biosynthesized, when sulfur concentration is enhanced (Figure 25). However, further increase had only minor effects. Thus, we could deduce that about 8 mM SO_4^{2-} is sufficient for maximal GS synthesis.

Interestingly the observed effects of sulfur supply do not apply for GN accumulated in the roots (Figure 26). The question arose, what could be the reason for this different pattern of GN accumulation? In principle there are two possibilities:

1. Synthesis of SI in the leaves and GN in the roots is catalyzed by two slightly different P450 cytochromes one producing SI and one GN, and these enzymes are differentially regulated depending on sulfur availability. Additionally, there could be differences in the supply of GS precursors in roots and shoots.
2. Biosynthesis of both SI and GN occurs in the leaves, and GN is then translocated subsequently. In order to elucidate this complex issue, much more experiments are required. Nevertheless, the sulfur depending differences in the accumulation of SI and GN could provide a valuable tool to get more information on the putative translocation of GS.

As the enhancement of sulfur supply from 1.7 to 8.3 mM resulted in a corresponding increase in GS concentrations, but a further enhancement to 21.5 mM SO_4^{2-} had no significant effects, the standard concentration for sulfur in the medium to investigate GS accumulation in horseradish *in vitro* plants was set to 8.3 mM SO_4^{2-}.

Nevertheless, the impact of sulfur enhancement on GS accumulation cannot explain the generally far lower concentrations of GS in *in vitro* plants compared to plants cultivated in the field. Accordingly, another issue must be responsible. It was argued that sulfur absorption from the media might be limited due to weak root system of the *in vitro* plants. Thus, an investigation of the root system on GS accumulation had to be conducted.

4.1.6. Role of the root system in sulfur absorption and subsequent effects on glucosinolate accumulation in horseradish *in vitro* plants

As outlined above, the second possible reason for the observed low GS accumulation in *in vitro* horseradish plants could be due to the fact that sulfur uptake might be limited due to the relatively weak root system of the *in vitro* plants. The root system development of *in vitro*

plants in general is affected by different factors. Yet, the most important ones are corresponding growth regulators and hormones, especially the presence of auxins. In order to elucidate, if the root system indeed is limiting sulfur uptake from the medium and thus responsible for the low GS synthesis, the root system was modified. For this *in vitro* horseradish plants were cultured on MS media and supplemented with different amounts of auxins. According to similar attempts, not the natural auxin indole acetic acid was applied, but the synthetic one, i.e., naphthalene acetic acid (NAA). For standard MS media, 0.5 mg/L NAA was supplemented. 1.0 mg/L NAA was added to enhance roots growth and to investigate its impact on GS accumulation. A comparison of plants cultured on these media is presented in Figure 27. As expected, root development is increased in response to NAA enhancement in the media.

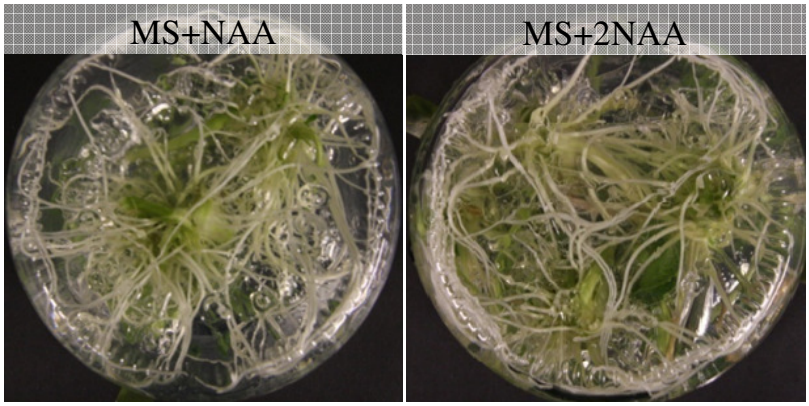


Figure 27: Comparison of horseradish *in vitro* plants grown on MS media supplemented with 0.5 or 1.0 mg /L NAA.

Although root formation was improved in plants cultured on media with doubled NAA concentration, SI and GN concentrations in leaves, stems and roots of *in vitro* plants did not increase (Figure 28). Astonishingly, the opposite effect was observed: Plants grown under enhanced NAA revealed in general slightly lower GS concentrations, although NAA supplementation enhanced the growth of the root system of *in vitro* plants. Accordingly, one may deduce that the reason for the lower GS concentrations of *in vitro* plants could not be caused by an insufficient capacity of the roots to take up the required amount of sulfur.

The data mentioned so far, clearly show that media composition, e.g., sulfur supplementation, is one factor, which limits GS accumulation in horseradish *in vitro* plants, however, the root system has no effects on GS accumulation. Accordingly, the major differences between total GS in *in vitro* plants and soil grown plants must be due to other factors.

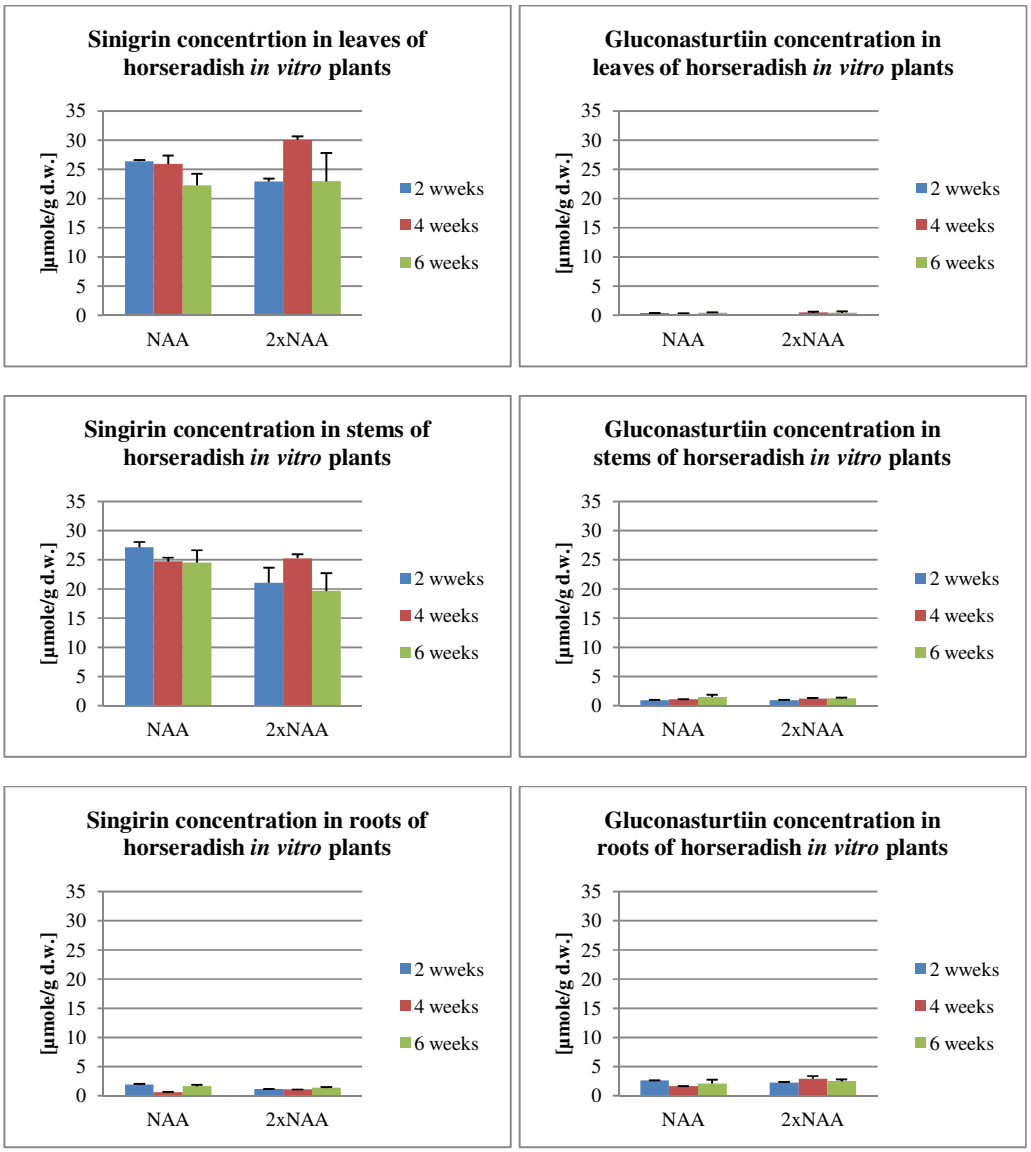


Figure 28: SI and GN concentrations in leaves, stems and roots of horseradish plants subjected to 0.5 mg/L NAA or 1.0 mg/L NAA over six weeks of treatment.

Putative other reasons might be the phytohormones presented in the medium. Many plant hormones and growth regulators plays a major role in regulating GS synthesis, in which jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and the stress hormone abscisic acid (ABA) have major roles (Mewis et al., 2006). However, other plant hormones might interfere with GS accumulation in plants; foliar application of indole acetic acid (IAA) to *B. campestris* was found to decrease GS in seeds, while seeds soaked in IAA solution showed an increase in GS accumulation (Bano et al., 2009). Moreover, IAA decreased 3-indolylmethyl glucosinolates in injured *B. napus* cotyledons, but its effect was insignificant (Bodnaryk, 1994). An increase of NAA concentrations from 0 to 1 ppm caused an

insignificant decrease in sulphoraphane concentration in *in vitro* cultivated broccoli sprouts during induction stage (Tilaar et al., 2012). Therefore, for the elucidation of the reason for the low concentrations of GS in *in vitro* plants, it will be necessary in further approaches to check the effects of growth regulators on GS synthesis to avoid any inhibition of GS accumulation. NAA was used due to its importance to enhance rooting in plants, and in conclusion, it turned out that the concentrations used with horseradish did not affect GS accumulation.

4.1.7. Effects of drought on glucosinolate accumulation in horseradish *in vitro* plants

Polyethylene glycol (PEG) restricts water absorption by the root system. Therefore, it could be used to induce drought stress in *in vitro* horseradish plants. The impact of drought stress on GS accumulation was investigated by applying various concentrations (0.0, 5.0, 10.0, and 15.0% (w/v); MW 20.000) of PEG.

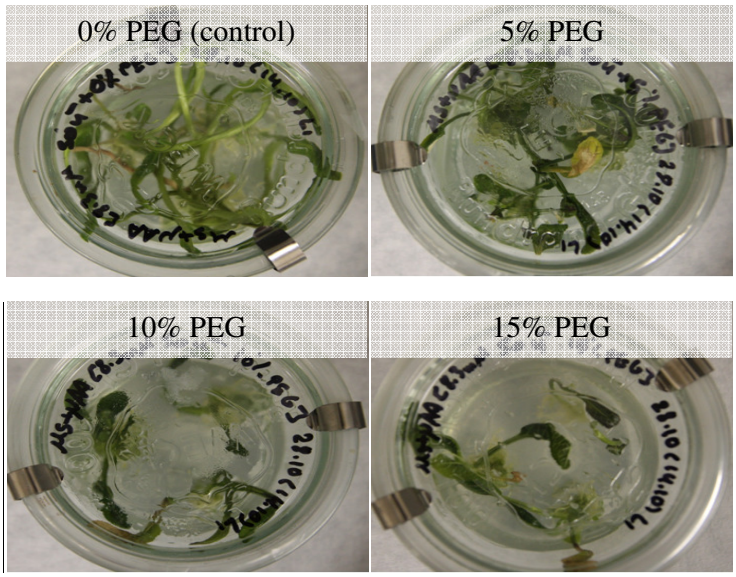


Figure 29: Comparison of plants cultured on media with various concentrations of PEG.

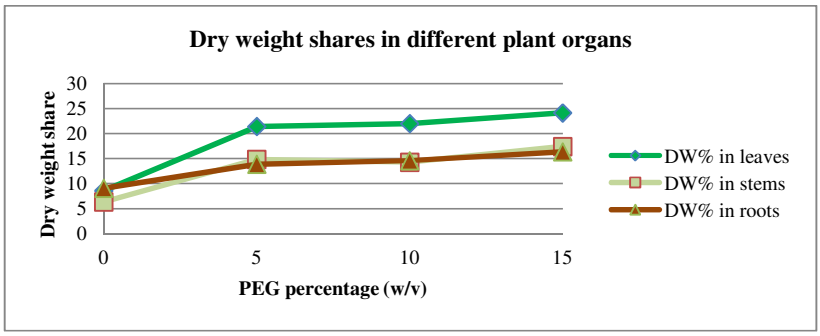


Figure 30: Dry weight share (DW%) in different plant organs (leaves, stems and roots) of horseradish *in vitro* plants after six weeks in response to different PEG treatments.

PEG treatments caused sound stress symptoms in *in vitro* plants (Figure 29). As compared to the control plants (0% PEG), plants suffering from stress showed retarded growth, and typical drought symptoms, such as wilting (Figure 29). Moreover, PEG treatments caused higher dry weight shares in all parts of horseradish *in vitro* plants compared to control plants. In general, leaves revealed the highest effect. Overall, a dry matter increase of about 15% was observed compared to plants cultured on media without PEG (Figure 30).

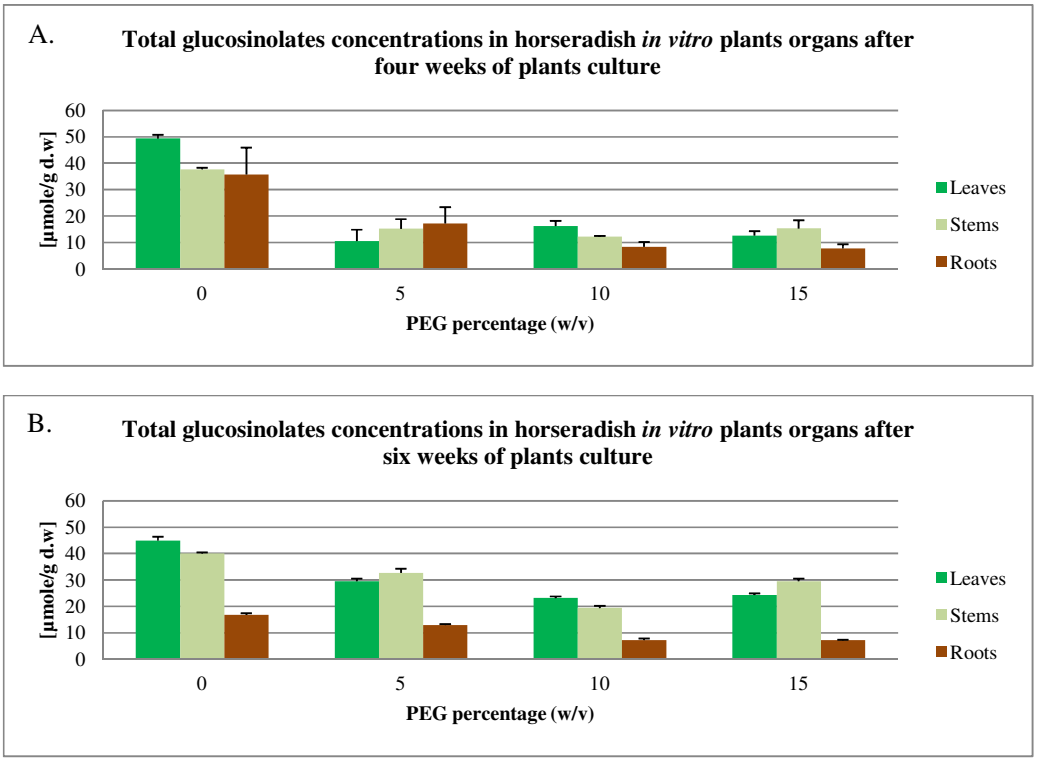


Figure 31: Total GS concentrations in horseradish *in vitro* plant leaves, stems and roots, after 4 (A) and 6 weeks (B) of cultivation on a media containing different PEG contents. Data represent duplicates, and bars resemble StD. Detailed data for SI and GN concentrations after 4 and 6 weeks are provided in appendices (Figure A2 and Figure A3, respectively).

Figure 31 shows total GS concentrations in different *in vitro* plant organs after 4 and 6 weeks of PEG treatments. Total GS concentrations in the organs of control plants are higher than total GS concentrations in the organs of plants cultured on media supplemented with different concentrations of PEG (Figure 31, A & B). Whereas this phenomenon is very pronounced after 4 weeks; after 6 weeks of cultivation, GS concentrations had increased to a much higher level. It seems that after 4 weeks of culture, plants had not well adapted to PEG containing medium, resulting in a much lower TGS concentrations after 4 weeks of culture. However, at

the end of culture period, plants accumulated higher total GS, which indicates that the plants have been better acclimatized to PEG containing culture medium.

Leaves, stems and roots of *in vitro* plants contain different concentrations of different total GS. Moreover, they contribute differently to plants biomass. Therefore, in order to get a clearer view of plant response to PEG treatment, total GS concentrations in horseradish *in vitro* plants on dry matter basis after six weeks of PEG treatments were calculated on whole plant basis (Figure 32). Control plants accumulated the highest total GS concentrations, followed by plants cultured on media supplemented with 5% and 15% (w/v) PEG, respectively. Plants cultured on media supplemented with 10% PEG (w/v) accumulated the lowest total GS concentrations (Figure 32).

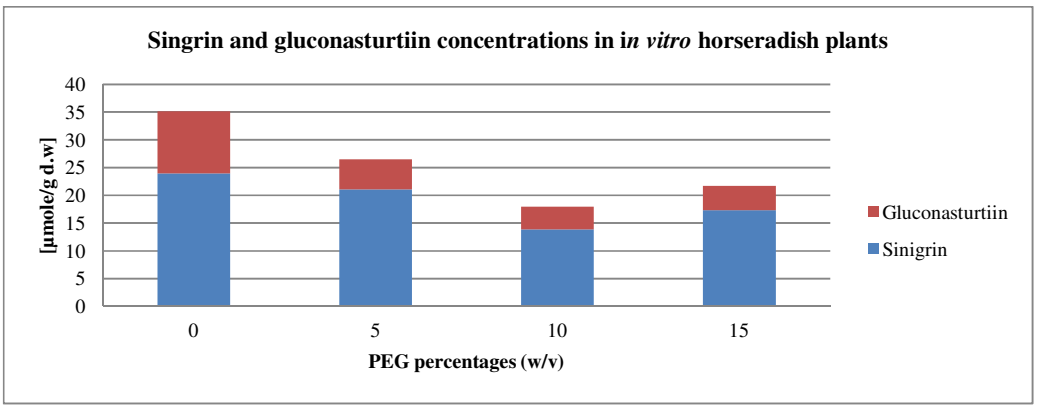


Figure 32: SI and GN concentrations in *in vitro* horseradish plants (on whole plant basis) after six weeks of treatments with PEG. Calculations for total GS concentrations on whole plant basis were conducted as described in section 3.7.2.

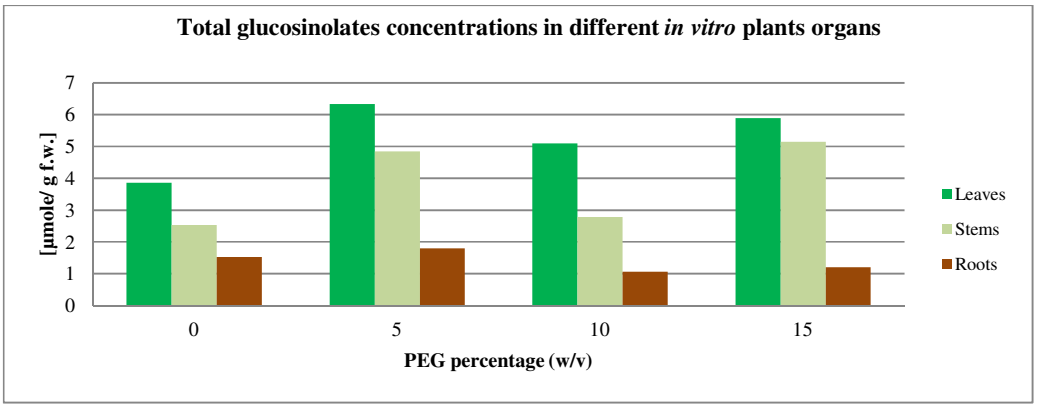


Figure 33: Total GS concentrations in leaves, stems and roots of *in vitro* plants on fresh weight basis after six weeks of plants culture on media containing PEG. Detailed data of SI and GN concentrations in various *in vitro* plants organs on fresh weight basis are presented in the appendix (figure A4).

According to our hypothesis on the stress enhanced biosynthesis of secondary plant products, this pattern of GS accumulation was not expected for plants suffering from drought stress. However, we have to take into consideration that the dry weight shares of the plants exposed to drought stress was quite different compared to the control plants. Thus, data were re-calculated on fresh weight basis, this represents the total GS concentrations in the living plants, and thus represent more actual plant physiological state.

The recalculation revealed a complete different pattern of total GS accumulation. Total GS concentrations were higher in the organs of the *in vitro* plants cultured on media containing PEG (Figure 33). The same pattern for total GS accumulation was also obtained when total GS concentrations were calculated in the whole plant on fresh weight basis (Figure 34). Obviously, the stress induced loss of water and the resulting increase in dry weight share compensates any changes in total GS. From these data, it could not be deduced wether, an increase or a decrease of GS accumulation in response of the PEG treatments occurred.

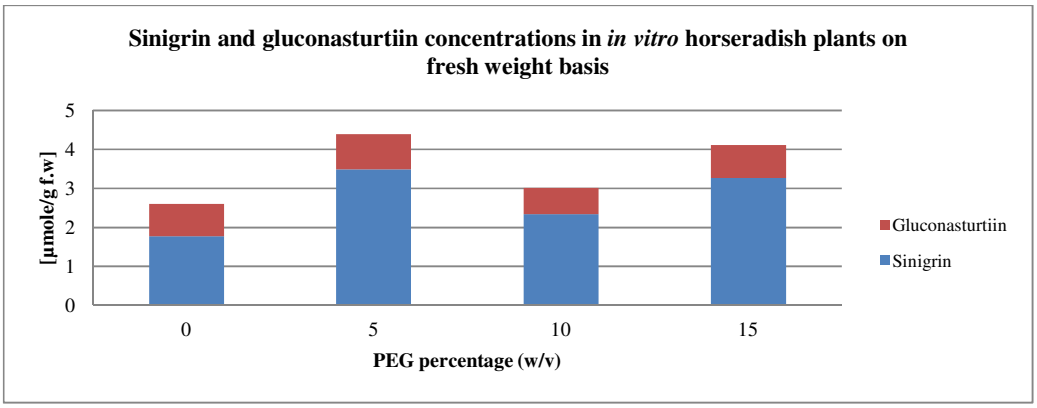


Figure 34: SI and GN concentrations in horseradish *in vitro* plants on fresh weight basis after six weeks of treatments. Calculations for total GS concentrations on whole plant basis were conducted as described in section 3.7.2.

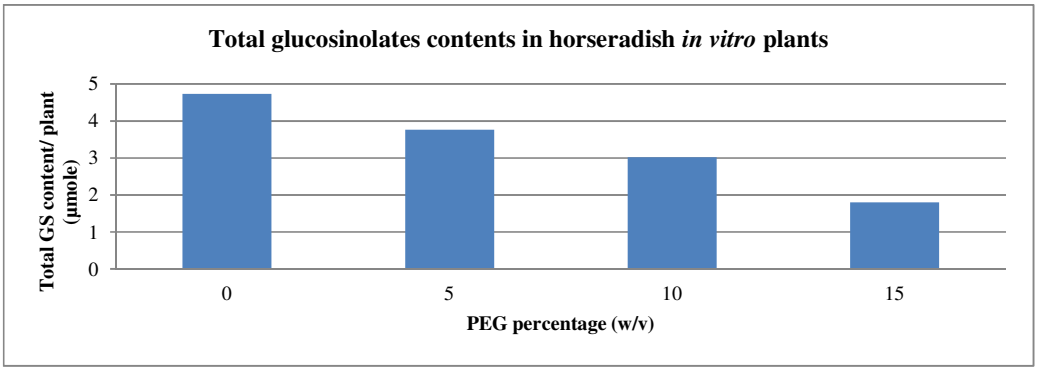


Figure 35: Total GS contents in horseradish *in vitro* plants subjected to various PEG concentrations.

For this, the total GS content had been calculated. Yet, due to the approach of total GS determination in individual organs, only rough estimates can be done on whole plant basis. Results obtained here show that the observed increase of total GS on fresh weight basis completely is overcompensated by the loss in biomass. Thus, plants cultured on media supplemented with PEG accumulated lower contents of total GS (Figure 35).

In order to elucidate a putative relation between drought stress and GS accumulation, an estimation of plant physiological status in response to PEG treatments is required. This will help in linking GS accumulation to changes in plant metabolism caused by different stress treatments. Since GABA accumulation as sensitive stress indicator is well documented (Bown and Shelp, 1997; Shelp et al., 1999), GABA concentrations were measured to evaluate the effect of different treatments on *in vitro* plants physiological state and corresponding changes in plant metabolism could be linked to possible effects on GS accumulation.

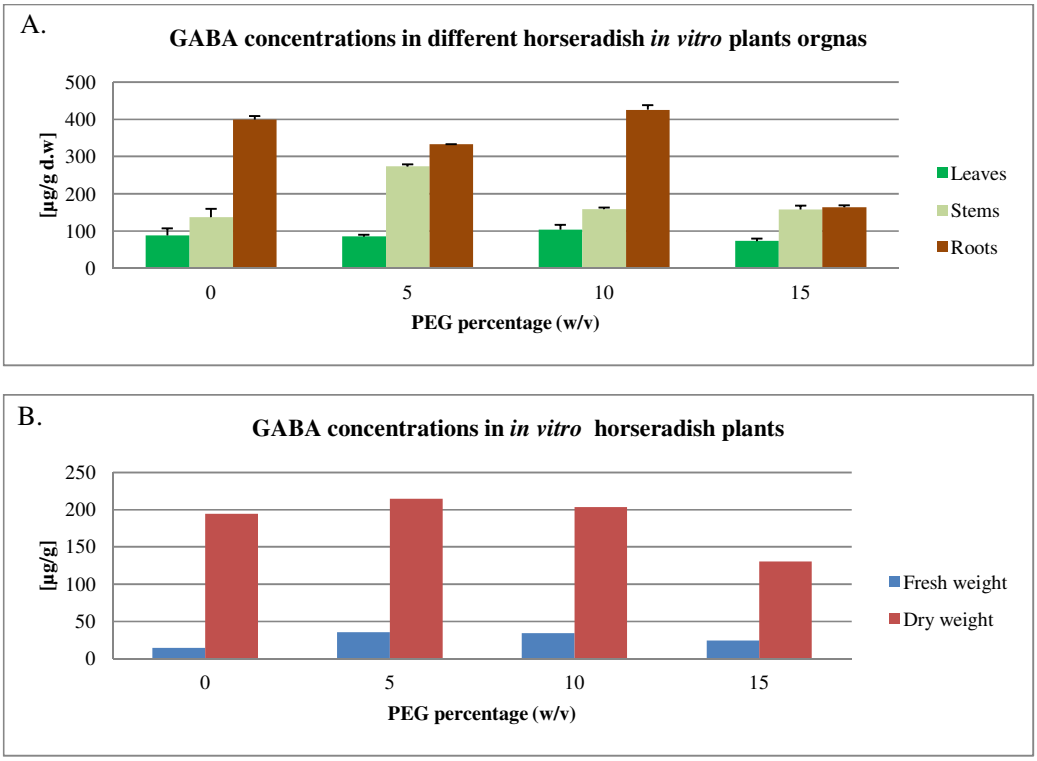


Figure 36: Concentration of GABA in horseradish *in vitro* plants. (A) In Leaves, stems and roots on dry matter basis, and (B) in the whole plant on dry and fresh weight basis, after six weeks of different PEG treatments. Bars resemble StD of double estimations. Calculations for GABA concentrations on whole plant basis were conducted as described in section 3.7.2.

GABA concentrations varied in the different *in vitro* plants parts in response to PEG treatments (Figure 36, A). In order to obtain a comprehensive overview of GABA concentrations, their concentrations in whole plant were calculated. GABA concentrations increased in response to moderate PEG treatments (5 and 10% (w/v)), then decreased slightly at high PEG treatment (15% (w/v) PEG) to levels lower than that found in the control plants (Figure 36, B). The same was observed on fresh weight basis. However, GABA concentrations in plants cultured on 15% (w/v) PEG, still revealed to be higher than that of the control plants (Figure 36, B).

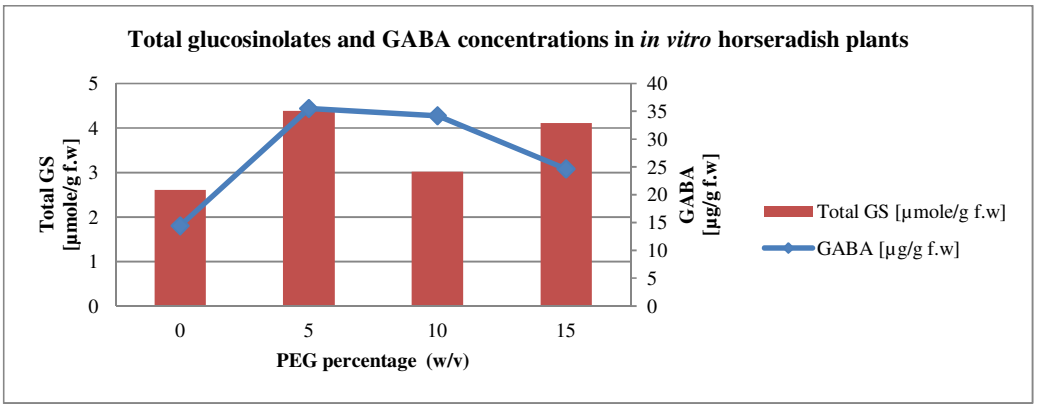


Figure 37: Total GS and GABA concentrations on fresh weight basis in *in vitro* horseradish plants after six weeks of PEG treatments.

In order to compare the appearance of the stress indicator GABA, with the changes in total GS concentrations, both parameters were combined in one graph. Due to the fact that the dry matter content changed drastically, corresponding data had been presented on fresh weight basis (Figure 37). Total GS and GABA concentrations in *in vitro* plants in relation to PEG treatments show similar patterns. Both increased in plants cultured on media containing PEG. In the case of plants cultured on 15% (w/v) PEG containing media, total GS concentrations increased while GABA concentrations decreased (Figure 37).

Discussion

PEG with high molecular weight, i.e., MW 20.000, was chosen to induce drought *in vitro*, since it allows similar osmotic control as the widely used PEG with smaller molecular weight (Williams and Shaykewich, 1969; Michel and Kaufmann, 1973). However, since high molecular weight PEG is not taken up by the plants, it does not interfere with plant

physiological processes (Lagerwerff et al., 1961) as low molecular weight PEG, and other low molecular weight osmolytes (Lawlor, 1970).

Similar effects of PEG on dry weight accumulation to those presented in this work were reported for barely leaves (Kadlecová et al., 2000) and for *A. thaliana* treated with PEG (Jing et al., 2010), and an increase in dry matter accumulation was also reported for five lotus species subjected to drought (Díaz et al., 2005a).

Patterns of total GS accumulation after four and six weeks of culture on PEG containing media are different from total GS accumulation pattern expected in plants subjected to drought stress (Figures 31). The same was also observed when total GS concentrations were calculated for whole plants on dry matter basis (Figure 32). This is different from the previously reported GS concentrations increase in *A. thaliana* in response to PEG treatments (Jing et al., 2010). Total GS concentrations are supposed to increase in response to drought conditions. This should aid in alleviating redox stress effects on plants, which resulted from water limitation caused by PEG application (Selmar and Kleinwächter, 2013; Wilhelm and Selmar, 2011).

Differences in dry weight shares affected total GS accumulation markedly, both in individual organs and on whole plant basis. The results obtained for total GS accumulation on fresh weight basis confirmed previous reports, in which plants challenged by drought accumulated higher GS concentrations compared to control plants (Jing et al., 2010; Gershenzon, 1984; Jensen et al., 1996).

Since GABA accumulation as sensitive stress indicator is well documented (Bown and Shelp, 1997; Shelp et al., 1999; Selmar and Kleinwächter, 2013; Kramer et al., 2010; Bor et al., 2009). GABA concentrations were measured to evaluate the effect of different treatments on *in vitro* plants physiological state.

GABA is known to be synthesized by several isozymes in shoots and roots, which might caus the observed fluctuation of GABA concentrations among different plant parts. Moreover, GABA possesses the ability to be mobilized between organs in the plant (Fait et al., 2008; Shelp et al., 1999).

GABA concentrations were calculated on whole plant basis to obtain an overview on the plant physiological state, and to link any observed changes with changes in GS accumulation

if possible. GABA concentrations on whole plant basis increased in response to mild stress, then decreased slightly at higher stress levels. Decreases in GABA contents might be explained by its role in nitrogen (N) and carbon (C) flux and storage. Therefore, GABA might be mobilized by the GABA shunt at high PEG concentrations to provide the plant with C and N required for vital processes (Bown and Shelp, 1997; Bouche and Fromm, 2004). Moreover, the decrease in GABA concentrations at the highest PEG treatment might be due to damage of plants biosynthetic machinery caused by severe drought. Such effect was reported earlier in plants subjected to drought caused by PEG treatments, and is characterized by a loss of turgor pressure of stressed plants leaves (Lawlor, 1969). This is consistent with the sharp increase of dry weight share in plants subjected to 15% (w/v) PEG (figure 30).

GABA increase in response to drought was also reported, significant differences in GABA concentrations were found in sesame plant leaves subjected to different treatments, i.e., control, 7 and 21 days of 15% PEG 6000 containing growth medium (Bor et al., 2009). Similar results were found in xylem sap of chickpea plants subjected to drought for 12 days. Moreover, a co-relation was found between GABA concentrations increase in leaves, roots and nodules in relation to the relative water content decrease in leaves of two chickpea cultivars, resulting from soil dehydration (Serraj et al., 1998).

Total GS and GABA seem to be affected by stress signals induced by drought stress. Total GS and GABA increases are thought to help in relieving stress caused by drought (Bouche and Fromm, 2004; Selmar and Kleinwächter, 2013; Schreiner et al., 2009). Therefore, both compounds show similar accumulation patterns. However, total GS and GABA are sensitive -at least in part- to different signaling pathways. Total GS synthesis is majorly regulated through SA and JA/ET signaling pathways (Mikkelsen et al., 2003; Mewis et al., 2005), which might be cross-influenced by ABA. On the other hand, GABA accumulation is sensitive to Ca^{2+} accumulation and changes in cytosolic pH, which are caused by drought and ABA signaling (Shelp et al., 1999; Gehring et al., 1990).

It seems that under mild stress, plant metabolism is active, and can accumulate different compounds to relieve stress. Under higher stress levels caused by elevated PEG concentrations (10% (w/v)), enhanced plant metabolism of certain stress metabolites, i.e., GABA occurs. At extreme stress conditions, plant metabolic machinery seems to be damaged. Under such conditions, GABA could also be mobilized through GABA shunt to provide plant with necessary elements, resulting in lower GABA concentrations. Moreover,

glutamate decarboxylase (GAD) activity is decreased at high cytosolic pH, which is caused by drought through ABA signaling (Bown and Shelp, 1997; Bouche and Fromm, 2004; Gehring et al., 1990; Shelp et al., 1999). Alternatively, plant metabolism might also shift to other types of solutes, such as reduced sugars to reduce severe osmotic effects caused by drought (Hoekstra et al., 2001).

Retarded growth of drought stressed plants, resulted in huge biomass differences. These differences were not compensated by high total GS concentrations accumulated in plants subjected to drought. Therefore, observed differences in total GS concentrations might be influenced by different degrees of vegetative growth.

In conclusion, drought induced by PEG enhanced total GS concentrations in horseradish *in vitro* plants. This increase in total GS levels can be monitored by elevated GABA concentrations, which also reflected plant physiological state.

Similar to drought, salinity limits water supply to the plant. However, the mechanisms of plant responses to adapt with water limitations caused by the two types of stresses are different. Moreover, salinity has lower effects on vegetative growth of plants compared to drought. Thus, it is of great importance to investigate if GS contents can be modulated, and enhanced upon plants challenge with salinity.

4.1.8. Salinity effects on glucosinolate accumulation in horseradish *in vitro* plants

Salinity limits water supply to plants, yet in general it has less effects on plants vegetative growth compared to drought stress. Therefore, in addition to drought stress, salinity was also applied to modulate GS content in *in vitro* plants.

Generally, the major factor responsible for inducing salinity is NaCl. Accordingly, in order to study salinity effects on GS accumulation in horseradish *in vitro* plants, MS media containing four different concentrations of NaCl (0, 25, 50 and 100 mM) were prepared.

Although NaCl treatments caused a clear reduction in vegetative growth (Figure 38), the overall habitat of salinity stressed plants was less affected by stress treatments compared to that of plants subjected to drought. Moreover, NaCl treatments have only limited effect on dry weight share in the various organs of horseradish *in vitro* plants. Among all plant organs, roots seem to be the most affected by NaCl treatments (Figure 39).

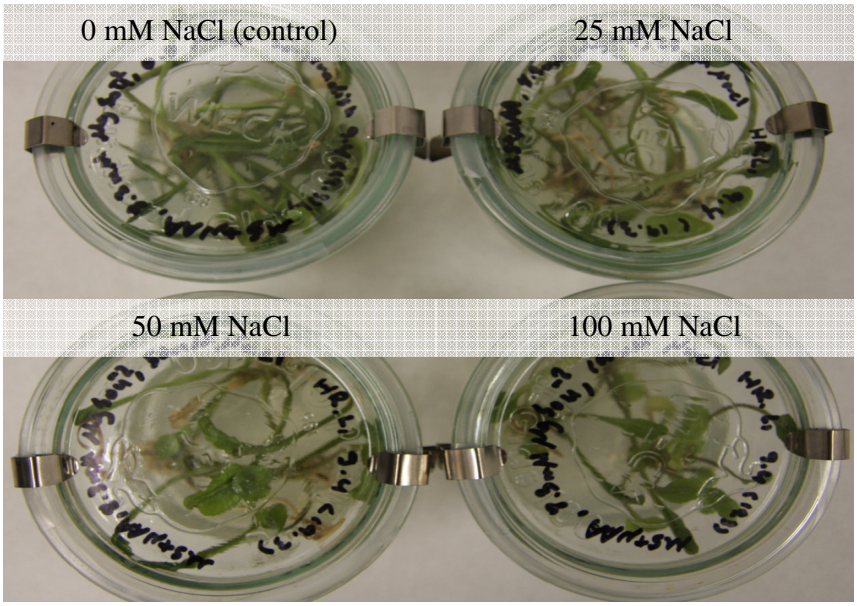


Figure 38: Comparison of horseradish *in vitro* plants cultured under various concentrations of NaCl.

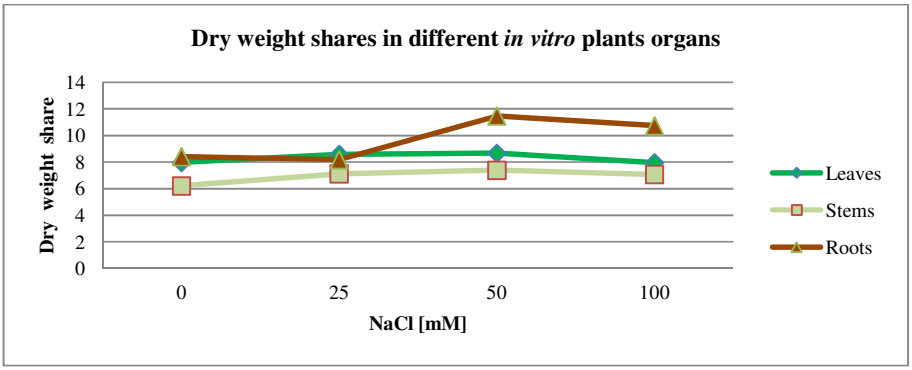


Figure 39: Effect of NaCl treatments on dry weight shares of horseradish *in vitro* plants (leaves, stems and roots) after six weeks of plants culture on 0, 25, 50 and 100 mM NaCl.

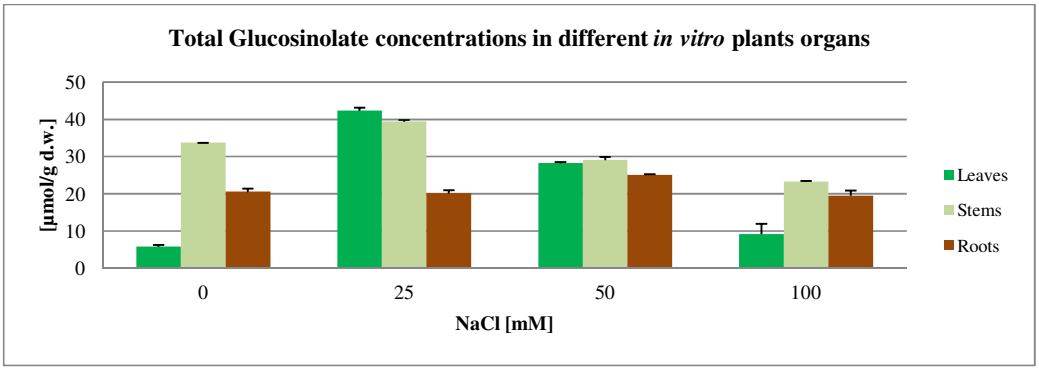


Figure 40: Effects of NaCl treatments on total GS concentrations in different horseradish *in vitro* plants organs after six weeks of NaCl treatments on dry matter basis. Bars resemble StD of double estimation. Detailed data for SI and GN concentrations are provided in the appendix (Figure A5).

After six weeks of NaCl treatments, concentrations of total GS increased in response to moderate NaCl treatments in all plant organs. Due to unclear reasons, control plants leaves contain very low amounts of total GS compared to the control plants from other experiments (Figure 40, for comparison see Figure 31, B). This result was not expected since leaves of control plants from other *in vitro* plants experiments revealed about 4-folds higher total GS concentrations. However, it should be kept in mind that beside various stress treatments many other factors affect GS accumulation. Moreover, other aspects of GS, such as translocation or remobilization might occur under certain conditions.

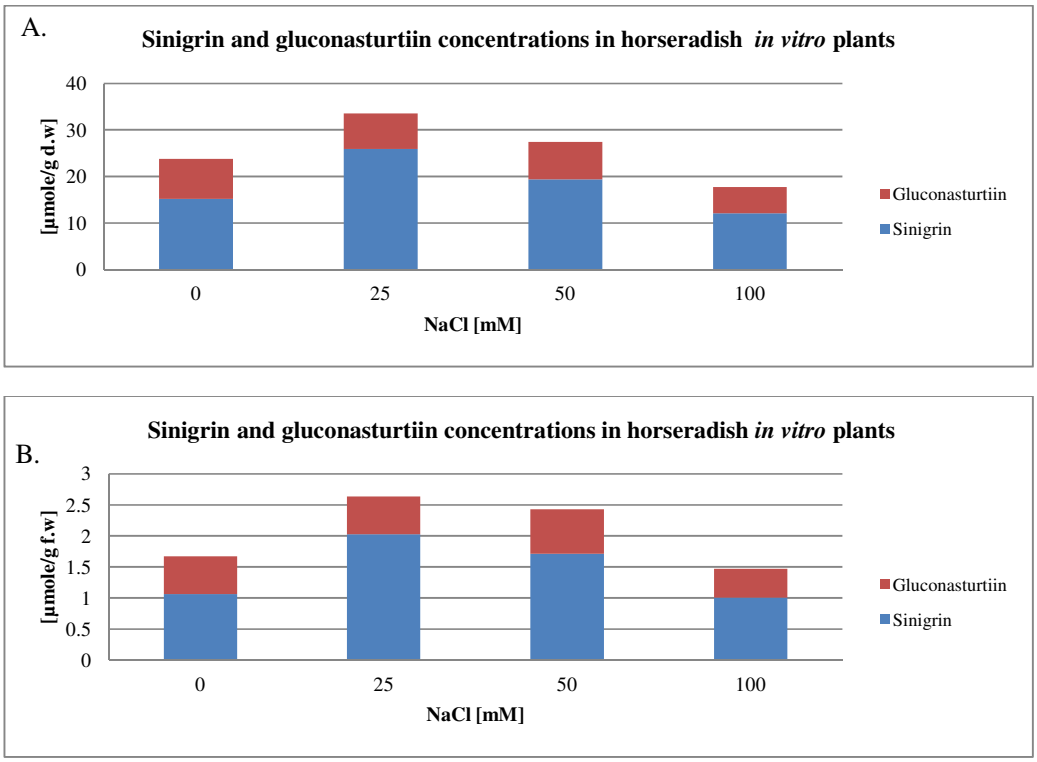


Figure 41: Concentrations of total GS (SI and GN) in *in vitro* plants on dry matter basis (A) and on fresh matter basis (B) after six weeks of NaCl treatments (0, 25, 50 and 100 mM). Calculations for total GS concentrations on whole plant basis were conducted as described in section 3.7.2. Details for SI and GN concentrations on fresh weight basis are provided in the appendix (figure A6).

For a comprehensive overview of the effect of salt on total GS accumulation, total GS concentrations were calculated in the whole plant on dry matter as well as on fresh weight basis. Total GS concentrations increased in response to moderately elevated salt concentrations (25 mM) as compared to control plants. Further enhancement of NaCl concentrations resulted in a decrease in total GS concentrations (Figure 41).

In contrast to the drought stress experiment (chapter 4.1.7), due to unchanged dry matter share, total GS accumulation pattern in horseradish *in vitro* plants after six weeks of NaCl treatments were not changed, when calculated on fresh weight basis (Figure 41, A & B).

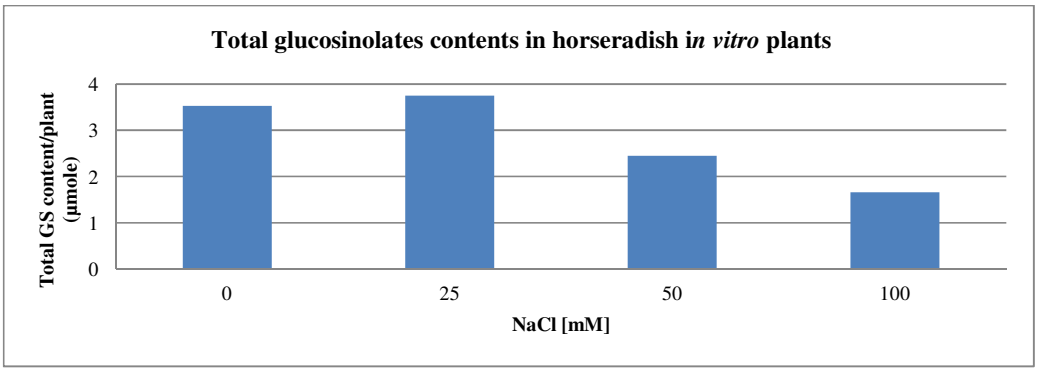


Figure 42: Total GS contents in horseradish *in vitro* plants cultured on media with various concentrations of NaCl.

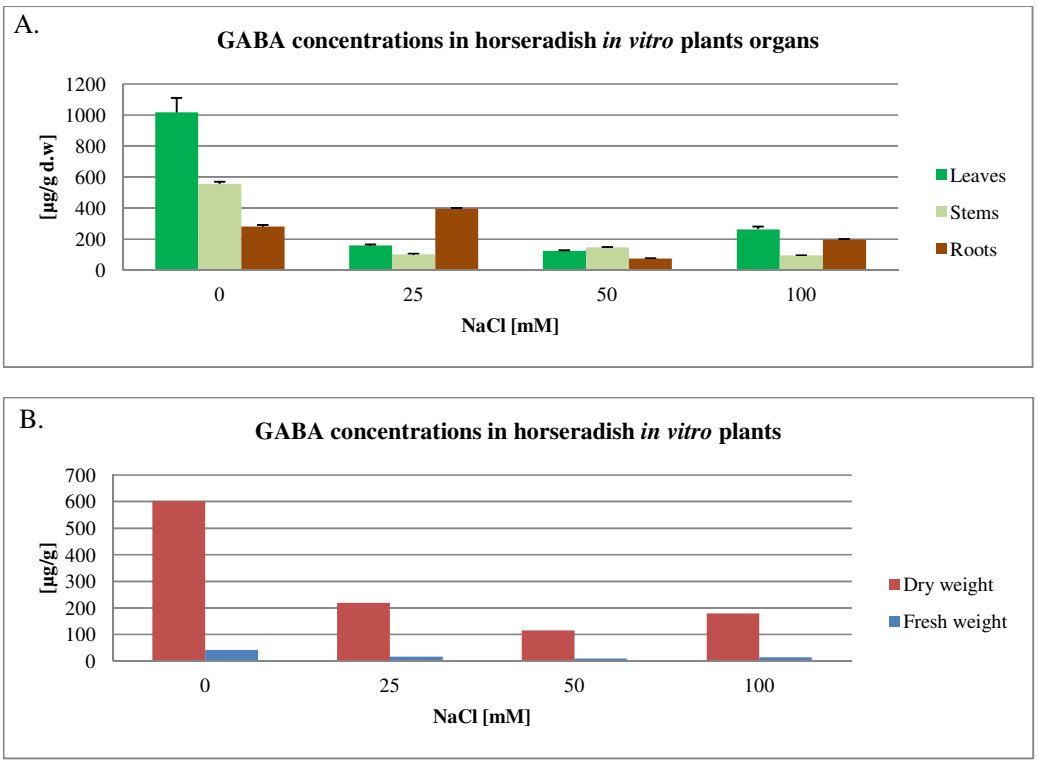


Figure 43: (A) GABA concentration in leaves, stems and roots of horseradish *in vitro* plants. (B) GABA concentration in horseradish *in vitro* plants after six weeks of NaCl treatments. Data for GABA concentrations in *in vitro* plants organs represent two separate extractions and analysis, and bars resemble StD. Calculations for GABA concentrations on whole plant basis were conducted as described in section 3.7.2.

Total GS contents in *in vitro* plants were also estimated on total plant base. Plants challenged with moderate salt concentrations (i.e., 25 mM) accumulated slightly higher total GS compared to control plants (Figure 42). Despite the small differences in contents, these results indicated that total GS accumulation can be modulated and enhanced to higher levels as compared to the control plants. However, further increases in NaCl concentrations decreased total GS contents, probably due to damage in plant metabolic machinery. Alternatively, cellular metabolic processes might shift to sustain vital metabolic functions of the plants. These results showed that only at moderate stress total GS contents can be enhanced, and further increases in salt concentrations applied affected GS accumulation negatively.

For the evaluation of the plants physiological state, also in this approach GABA concentration as general stress indicator was determined. GABA concentrations in horseradish *in vitro* plants leaves, stems and roots after six weeks of NaCl treatments are presented in Figure 43, A. The organs of the control plants revealed higher GABA concentrations compared to the NaCl treated plants (Figure 43, A). This result is surprising, since the GABA concentration in the leaves is about 10-fold higher than in the leaves of the corresponding control plants of the drought stress experiments. We have to consider that apart from the stress situation applied by drought and salinity, quite other factors are influencing GABA accumulation (Bouche and Fromm, 2004). GABA accumulation pattern was not changed by calculating the concentrations on whole plant on dry and fresh weight basis (Figure 43, B). Therefore, our findings showed that GABA concentrations are not a suitable marker for salinity stress in horseradish *in vitro* plants.

Discussion

The finding that NaCl treatments affect only slightly dry matter share of leaves and stems is in agreement with data reported previously of NaCl effect on relative water content of two broccoli cultivars (Zaghdoud et al., 2012). Almost no change was observed between control plants, and plants subjected to 90 mM NaCl (Zaghdoud et al., 2012). On the other hand, data obtained for roots differ from those reported for the broccoli cultivars, which show small changes in relative water content (Zaghdoud et al., 2012).

The pattern of total GS accumulation is similar to that found by Keling and Zhujun, (2010) in *Brassica campestris* plants grown in greenhouse. Total GS concentrations increased in shoots

of *B. campestris* when subjected to 50 mM NaCl but decreased upon treatment with 100 mM NaCl (Keling and Zhujun, 2010). Different broccoli cultivars responded differentially to NaCl treatments, in "Parthenon" (widely used cultivar) indole and total GS increased in response to 30 mM NaCl but decreased when subjected to higher NaCl concentrations (60 and 90 mM NaCl). In contrast, both types of GS decreased in response to increase in NaCl concentrations in Naxos cultivar, which is known for its high tolerance against abiotic stress (Zaghdoud et al., 2012). On the other hand, aliphatic GS increased in response to increased NaCl concentrations in both cultivars (Zaghdoud et al., 2012).

Results obtained for GABA accumulation differ from that obtained previously, in which the authors show that GABA concentrations increased in response to NaCl treatments of *Arabidopsis* and sesame (Renault et al., 2010; Bor et al., 2009). However, both groups used 150 mM NaCl, which is higher than the concentrations used in this experiment. Moreover, the data of the *Arabidopsis* experiment were obtained over relatively short period; 8 days for GABA in whole plants, and 4 days for shoots and roots (Renault et al., 2010). These authors investigated also the activity of several glutamate decarboxylase (GAD) isozymes, the enzyme responsible for GABA formation by decaboxylating glutamic acid. The various isozymes revealed different activity responses to NaCl treatments (Renault et al., 2010). Thus, it seems that the individual isozymes response to NaCl plays a determining role in GABA accumulation in NaCl treated plants. Moreover, GABA catabolising enzymes, i.e., GABA transaminase (GABA-T) and succinate semialdehyde dehydogenase (SSADH), along with positive stress indicator genes were up regulated after 24 hours of NaCl treatment (Renault et al., 2010). Therefore, reported data might differ if measured at longer periods of treatment.

In contrast to plant responses to a wide variety of stresses, Ca^{2+} concentrations in plants cells subjected to salinity are decreased or remained constant. NaCl treatments cause an enhancement in Na^{+} concentrations inside the plant cells, which responded with pumping Ca^{2+} ions outside the cell to maintain ions balance for essential metabolic processes.

GAD activity increases in response to increased Ca^{2+} concentrations and at acidic pH (Shelp et al., 1999). However, NaCl treatments are not accompanied by changes in the cytosolic pH value, which has been reported for at least some species (Halperin et al., 2003). Therefore, GABA biosynthesis should not be increased under NaCl treatment, at least not theoretically. GABA accumulation is not solely controlled by GAD activity, but it is also affected by

catabolism and translocation from one organ to another. Moreover, GABA can be mobilized to provide the plant with nutrients under certain stressful conditions (Shelp et al., 1999). The sum of these factors might cause different estimations of GABA concentrations in plants.

Although GS and GABA concentrations are accumulated in response to wide variety of biotic and abiotic stresses (Bouche and Fromm, 2004; Shelp et al., 1999; Bown and Shelp, 1997; Selmar and Kleinwächter, 2013; Mewis et al., 2005), it seems that they are responsive -at least in part- to different physiological factors; GS biosynthesis is finely regulated by JA, SA and ET signaling pathways (Mewis et al., 2005). On the other hand GABA biosynthesis is affected by cytosolic pH and Ca^{2+} concentrations (Shelp et al., 1999). These differences might cause differences in GS and GABA responses and thus different accumulation patterns, as evident here in horseradish *in vitro* plants in response to NaCl treatments.

In conclusion, salinity enhanced GS accumulation in *in vitro* horseradish plants. However, GS accumulation was not accompanied by corresponding increases in GABA concentrations. GABA does not seem to be a suitable marker for salinity stress, at least for some plants, since it failed in reflecting plants physiological state.

Plants responses to abiotic stresses are mediated by many plants hormones. ABA is a key hormone in plant signaling in response to abiotic stress. Therefore, the effect of exogenously applied ABA on GS accumulation had been addressed.

4.1.9. Effect of abscisic acid on glucosinolate accumulation in horseradish *in vitro* plants

Abscisic acid (ABA) plays a major role in signaling pathways of plants suffering from abiotic stress, and the expression of many genes after exogenous ABA application overlaps with their expression due to abiotic stresses, such as salinity and drought. ABA majorly affects plant responses to water shortage by targeting guard cells to close stomata (Tuteja, 2007). Accordingly, exogenous ABA was applied to horseradish plants cultured *in vitro* in order to impact the synthesis and accumulation of GS.

ABA treatments affect the plant growth in the similar manner as drought. Generally, stressed plants were smaller and revealed retarded growth. These effects could be enhanced by increasing the concentration of ABA.

The corresponding experiment revealed that ABA treatments caused an increase in dry weight share of all plant parts; leaves were most affected by ABA treatments followed by stems and roots; their dry weight increased by about 18, 5 and 3%, respectively (Figure 44).

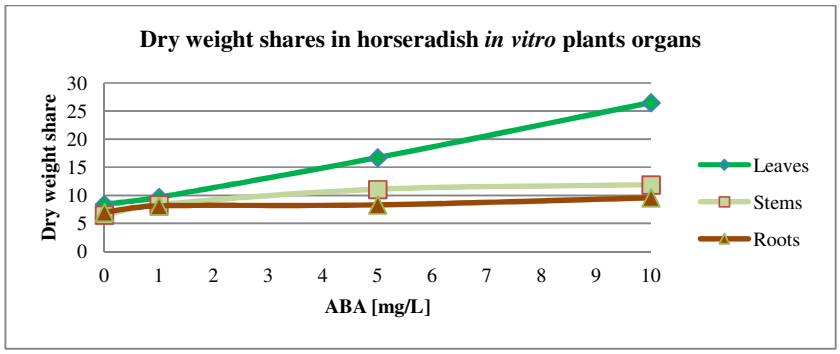


Figure 44: Dry weight share of leaves, stems and roots of *in vitro* horseradish plants after six weeks of ABA treatments.

In response to ABA treatments total GS concentrations decreased to levels lower than that of the control plants. Total GS concentrations were slightly lower when 1 mg/L ABA was applied as compared to 5 and 10 mg/L ABA treatments (Figure 45).

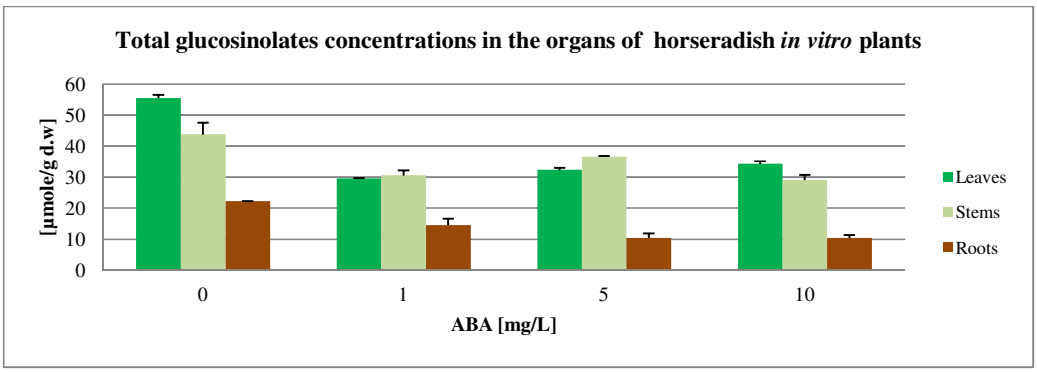
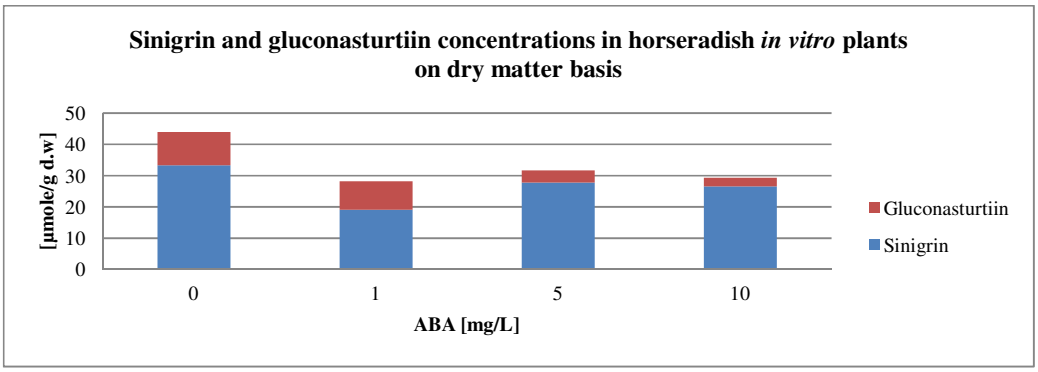


Figure 45: Total GS concentrations in the organs of horseradish *in vitro* plants after six weeks of ABA treatments. Data represent double estimations. Bars resemble StD. Detailed data for SI and GN concentrations are available in the appendix (Figure A7).

For further considerations, total GS concentrations in the plants treated with ABA were calculated for the entire plant on dry weight basis. All plants treated with ABA revealed lower total GS concentrations than the control plants (Figure 46, A). However, in this context we have to take into consideration that the dry weight shares strongly increase as result of ABA treatments (Figure 44). In order to eliminate this influence, total GS concentration was calculated on fresh weight basis. As assumed, the change of the basis caused a reversal of the

pattern (Figure 46, A & B). On the first glance, it might be deduced that ABA application indeed causes higher rates of GS biosynthesis and accumulation. However, it has to be considered that the biomass production is strongly diminished. Sound statements on the overall effect of ABA on GS metabolism only can be made, when the massive differences in biomass are considered adequately. Accordingly, the total contents of GS in the plants have been calculated. The striking results are presented in Figure 47: Although the concentrations of total GS increased significantly, the overall contents decreased as result of the ABA treatment. This means that it cannot be deduced that the GS biosynthesis is enhanced by ABA treatments. Moreover, effects of biomass differences on GS accumulation cannot be excluded.

A.



B.

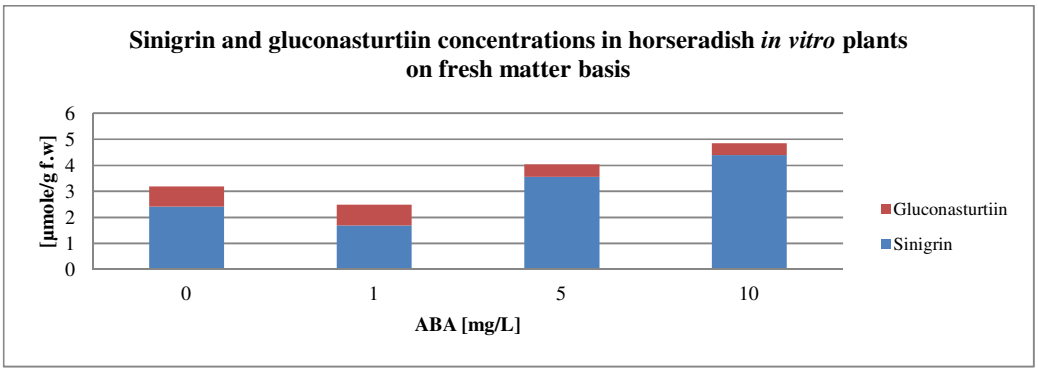


Figure 46: SI and GN (total GS) concentrations in horseradish *in vitro* plants after 6 weeks of ABA treatments on dry matter basis (A), and fresh matter basis (B). Calculations for total GS concentrations on whole plant basis were conducted as described in section 3.7.2. Details for SI and GN concentrations on fresh weight basis are provided in the appendix (Figure A8).

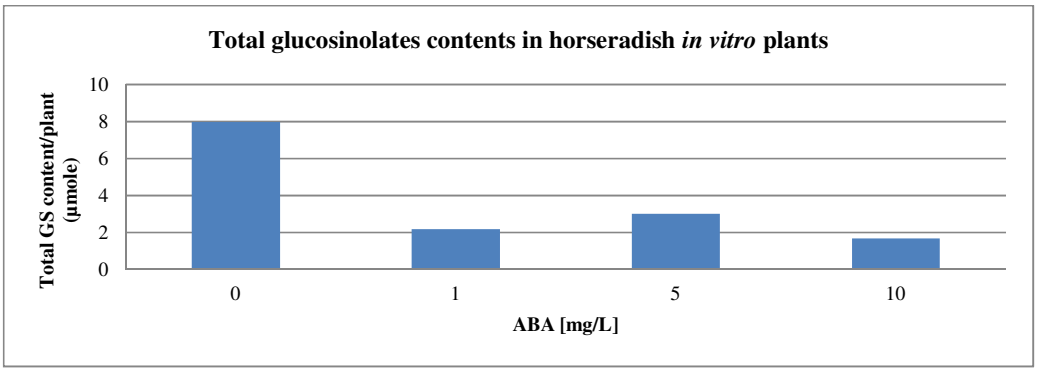


Figure 47: Total GS contents in horseradish *in vitro* plants after six weeks of ABA treatments.

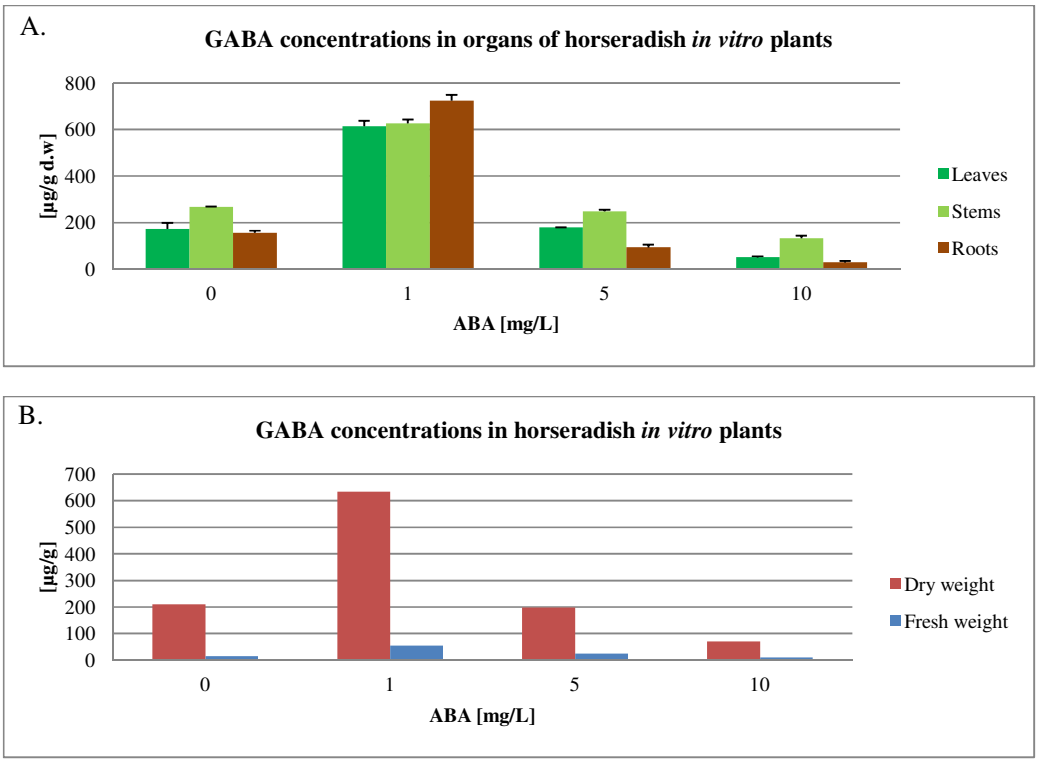


Figure 48: GABA concentrations in (leaves, stems and roots) of horseradish *in vitro* plants on dry matter basis (A), and GABA concentrations in whole horseradish *in vitro* plants on dry weight and fresh weight basis (B) after six weeks of ABA treatments. Bars resemble StD of duplicate estimations. Calculations for GABA concentrations on whole plant basis were conducted as described in section 3.7.2.

The physiological state of the plants was evaluated using GABA as stress indicator. The GABA concentrations increased sharply in all plant organs in response to 1 mg/L ABA, but decreased in response to higher ABA treatments (Figure 48, A). The same is true, when GABA concentrations were calculated for the whole plants on fresh and dry weight basis (Figure 48, B). Thus, GABA concentrations are a suitable marker for stress induced by

exogenous ABA treatments. However, GABA and total GS concentrations reveal different accumulation patterns. Therefore, GABA concentrations cannot be used as indicator for GS accumulation in horseradish *in vitro* plants treated with exogenous ABA.

Discussion:

Exogenous ABA treatments reveal short and long terms effects on plants. Short term effects of ABA occurr within 5 minutes in response to ABA. These effects include the control of stomata aperture to manage plant water status. Long term effects of ABA on plants reveal an impact on RNA and protein synthesis (Zeevaart and Creelman, 1988). Therefore, it is expected that ABA treated plants will not reveal different dry weight shares compared to the control at short term treatments. However, protein accumulation in the longer term could affect dry weight share. In addition, plants treated with exogenous ABA revealed similar morphological characteristics to plants suffering from drought stress (Walton, 1980). High dry matter share after application of ABA was observed in the horseradish *in vitro* plants used in this experiment. In accordance with the results obtained here, an increase in dry matter share (decrease in plant water content) was reported for several clover cultivars subjected to 1 mM ABA for 11 days (Xing et al., 2002).

Literature gives inconsistent data concerning GS accumulation in response to ABA treatments. Generally, GS concentrations were slightly affected upon the usage of low concentrations of ABA, e.g., GS decreased in seeds when ABA was used as foliar application at the vegetative phase (Bano et al., 2009). ABA treatment of microspore derived embryos of *B. napus* slightly increased GS concentrations, although it caused decreases in indole GS (Möllers et al., 1999). Moreover, ABA caused a slight increase of GS in *B. campestris* seeds, and an insignificant increase in *B. napus* cotyledons (Bano et al., 2009; Bodnaryk, 1994). In addition, a transcriptome analysis study in *A. thaliana* concluded that GS-related genes, which were up-regulated by ABA treatment, act in GS catabolism, diverting GS degradation to nitriles, which provide stressed plants with nitrogen and sulfur as argued by the authors (Böhmer and Schroeder, 2011). A proteome and metabolime study linked between aliphatic GS decrease in mutant *A. thaliana* plants and endogenous ABA increase (Chen et al., 2012). However, the authors mentioned that indolic GS concentrations were increased without giving detailed data, or relating this to ABA concentrations (Chen et al., 2012). Our results are in agreement with previous reports on the effect of low ABA concentrations, in which

total GS concentrations were slightly affected. This was manifested by a slight decrease in total GS concentrations compared to their concentrations in control plants.

In contrast to the data on the effect of low concentrations of ABA on GS accumulation, at high ABA concentrations, total GS concentrations increased in *A. rusticana.* Plants responses to biotic and abiotic stress are regulated by very complex signaling pathways, in which ABA plays a very important role. However, its exact functions needs to be further elucidated, especially in GS accumulation. Erb et al. (2011) argued that although ABA was induced in response to *D. virgifera* infestation of *Zea mays* plants, it has only a partial role in plant defense, while hydraulic changes in the leaves were crucial for plant resistance (Erb et al., 2011). Therefore, it seems that not only ABA concentrations cause GS concentrations to increase, but other factors involved in stress signaling have a crucial role in GS accumulation as well, and that is the reason why a synergetic effect of ABA dependent and independent mechanisms is expected to cause the observed increases in GS concentrations, after being decreased at low ABA concentrations (figure 46, B).

The total GS decrease in response to low ABA treatments might be due to increased resistance in plants upon ABA increase and subsequent stomata closure (Jakab et al., 2007), as well as due to negative mechanisms controlling stress signals emerged from ABA accumulation (Vadassery et al., 2012). However, at higher ABA concentrations and subsequent plant stress, the negative control mechanisms of ABA may be less effective, due to the accumulation of other ABA-dependent and ABA-independent stress signaling molecules, which cause an increase in GS accumulation.

GS synthesis is affected by various biotic and abiotic factors (Selmar and Kleinwächter, 2013; Mikkelsen et al., 2003), and their synthesis is regulated by different signaling pathways, in which JA, SA and ET play a major role (Mewis et al., 2005).

Abiotic stress responses are mediated –at least in part- by ABA, and endogenous ABA concentrations increase in response to different stresses (Vadassery et al., 2012; Jakab et al., 2007). The role of ABA in biotic stress is poorly understood, and it is discussed controversially. However, ABA seems to increase JA and thus plant resistance at least in response to some pathogens (Adie et al., 2007). Accordingly, ABA might affect GS synthesis by regulation of JA and SA concentrations. ABA suppressed indole GS accumulation, which was induced by JA in *B. napus* embryos (Möllers et al., 1999). Although, the GS

concentrations remained higher than in the control plants. The high ABA concentrations used here might cause the activation of GS synthesis by activating JA-related genes in a concentration dependent manner. Adie et al. (2007) interpreted that ABA levels might be responsible for the differential roles of ABA in plant-pathogen interactions. Moreover, ABA might increase GS synthesis by suppressing SA accumulation (Fan et al., 2009).

The GABA synthesis by glutamate decarboxylase (GAD) is sensitive to pH and Ca^{2+} concentrations (Shelp et al., 1999), both of them (pH and Ca^{2+} concentrations) are increased by ABA (Webb et al., 2001; Jakab et al., 2007; Gehring et al., 1990) but Ca^{2+} is increased faster than pH (Gehring et al., 1990). Ca^{2+}/calmodulin (CaM) interactions highly activate GAD activity. However, GAD activity is negatively regulated by pH increase.

At low ABA concentrations, as used in this experiment, GABA synthesis is increased by Ca^{2+} /CaM-activated GAD due to high accumulation of Ca^{2+}, while at higher pH caused by severe treatments, GABA synthesis is decreased due to GAD inhibition. GAD shows only ≈40% activity at pH 7.0. Moreover, GABA might be catabolized to provide the plant with nutrient under stressful conditions (Shelp et al., 1999), causing GABA concentrations to decrease.

Similar to osmotic stress, ABA causes stomata closure. However, sometimes this did not result in stress induction in plants. Accordingly, it is expected that stress induction and its subsequent manifestation depend on the pathway by which the stress is transduced. Signals of abiotic stresses are known to be transmitted by two pathways: ABA dependent and ABA independent. Accordingly, only part of the entire stress response is initiated in response to exogenous ABA treatments, and this was not sufficient to affect GS accumulation markedly.

It can be concluded, that similar to abiotic stresses, exogenous ABA caused an increase in GS concentration, but the pattern of GS accumulation is different from that observed earlier in response to drought and salinity. Due to huge differences in biomass, overall GS contents were decreased. Therefore, an enhancement of GS biosynthesis as a result of exogenous ABA treatments cannot be concluded. Moreover, GABA accumulated in response to ABA in a different pattern compared to GS. Thus, although GABA is considered to be a good indicator for the physiological state, it cannot be used to monitor GS concentrations in plants treated with exogenous ABA. The role of ABA on GS accumulation is very interesting and further investigations are required to decipher their interaction.

Future work on *in vitro* plants might consider higher light intensities. This will cause higher reductive stress, which might affect plant metabolism and GS biosynthesis. Moreover, studies focusing on stress conditions that result in higher accumulation of GS can be conducted separately or in combination. Finally, the control of biomass change in response to stress, and its subsequent effect on GS accumulation is highly important. The optimization of plant growth and GS biosynthesis is a key step for further experiments investigating plant quality improvement for both scientific and economical reasons.

From *in vitro* experiments, it is clear that GS concentrations can be modulated and enhanced by using exogenous abiotic factors. However, under the controlled environment of *in vitro* culture, single abiotic factors or stresses can be manipulated and applied easily. Under environmental conditions, mature plants cannot be cultivated at the same strict conditions, hence they are subjected to various kinds of stresses simultaneously. Interactions between various stresses might modulate the expected outcome of GS accumulation. Therefore, mature horseradish plants grown in a rain shelter or cultivated in the field were used. This will aid to further investigate the effects of abiotic stresses on GS accumulation as well as to sound out the feasibility of deliberately applying abiotic stresses to improve the quality of horseradish tubers.

4.2. Effect of exogenous factors on glucosinolate accumulation in horseradish plants grown under rain shelter

Horseradish mature plants were grown under a rain shelter to investigate the effect of abiotic stresses on GS accumulation. The rainshelter was created to protect plants from rainfall. Thus, the water supply to the plants can be regulated, and controlled drought conditions could be induced. Moreover, the horseradish plants were grown under conditions similar to naturally cultivated ones. Accordingly, the applicability of abiotic stresses for modulating GS contents can be investigated. This should aid in optimizing the conditions for producing horseradish tubers with enhanced qualities.

The various soils and organic matter generally used as plants growth substrate have different capacities to absorb water. Therefore, for controlled treatments, water amount used in irrigation and soil capacity to absorb it have to be determined in order to establish a sound experimental setup for investigating the effects of abiotic stress on GS accumulation.

4.2.1. Elaboration of growth conditions

Water holding capacity (WHC) was pre-estimated in order to determine the capacity of the used potting soil to absorb water. These data will be used to determine the water supply for the various treatments.

Table 13: Determination of WHC

Time (hour)	Pots weight after irrigation (kg±0.01)	
	Pot 1	Pot 2
1	6.66	6.67
2	6.64	6.66
3	6.63	6.64
4	6.62	6.63
5	6.61	6.62
6	6.61	6.61
7	6.6	6.6
8	6.59	6.6

Pots used to cultivate the plants were carefully chosen to retain water. Therefore, they have closed bottoms. In order to determine the amount of water, which could be present without causing backwater, WHC was determined. Thus, only absorbed water should be retained. For

this, several holes were manually drilled at the bottom of the used pots before filling them with potting soil.

WHC capacity was determined by saturating the used potting soil with water. Then, the weight of the pots was determined over eight hours time period. Water loss was stable after 6-8 hours of irrigation. From total weight of soil and retained water, maximum amounts of water for plants irrigation can be determined (Table 13).

The results obtained in the lab for WHC were quite similar, when soil samples were dried in an infra red balance; dry matter composed ≈29% of the sample weight. Then, 100 g of soil sample were saturated with water and placed in a glass dish that allows water percolation to a surrounding sandy soil. The water loss was recorded each 30 minutes over 7 hours period. WHC was calculated from fresh and dry weight of the used sample, when weight was almost stable (≈ 6 hours after saturation). 1 g of dried soil was found to retain 4.7 folds of water. The used 1.16 kg of dried soil were found to retain about 5.45 kg of water, thus soil and water weight was 6.61 kg.

In parallel, before growing the plants, water content was determined in pots irrigated with WHC using TDR probe, preliminary measurements of two pots gave similar results of water content, i.e., 36.6 % and 36.4% (v/v). Throughout the experiment, water contents of 10 individual pots of each treatment were measured 9 times for each pot, then average of similar treatments of different varieties, e.g., average of water content in plants receiving water amounts equal to WHC of East (E), Badisch (B) and Kröner (K) varieties, was determined, which is the average of 270 individual estimations. For simplicity these averages were used to demonstrate different treatments of this experiment (Table 14).

Table 14: Average of water contents measurements in different treatments of all varieties.

Treatments		**Average**	
		H_2O% (v/v)	**WHC%**
Excess of water	**WE**	38.99	100.00
Control	**C**	32.28	82.80
Moderate drought stress	**MS**	25.71	65.94
Severe drought stress	**SS**	22.55	57.83

In order to induce drought stress, water content in the soil was decreased. One of the first responses of plants subjected to drought stress is the closure of stomata. This can easily be determined by the reduction of transpiration. In the case of open stomata, transpiration could

not be enhanced by further increase of water supply. However, too high water concentrations could yield in deficiencies in the oxygen supply of the roots caused by backwater. When the transpiration of plants grown at 100% WHC is compared with that of plants cultivated at 80% (Table 15), it turned that at 80% WHC there is no change in stomata aperture. Moreover, their vegetative growth was very similar. Accordingly, a WHC of 80% was chosen as condition for the control plants (C).

In another approach, the water content was decreased to about 66% WHC (corresponding to about 25.7% water content ≈MS) or to about 58% WHC (corresponding to 22.5% water concentration ≈SS). Under MS conditions, transpiration declined to about 77.5% of the rate of the control plants, and under SS conditions down to 56.3% (Table 15). Thus, the stomata have partially been closed in the plants grown under MS conditions and they have been even more shut at SS conditions. Accordingly, in contrast to plants grown at control conditions, those from the MS approach should suffer moderate drought stress and those from SS massive drought stress. These conditions had been applied for the experiments to elucidate the impact of drought stress on the GS content. The differences in transpiration were maintained throughout the entire experiment.

Table 15: Transpiration of plants subjected to different watering treatments. Data are average of three plants.

Average	WE	C	MS	SS
WHC%	**100%**	**82.8%**	**65.9%**	**57.8%**
Transpiration (ml)	130.0	131.7	96.7	80.0
	180.0	163.3	128.3	73.3
	256.7	258.3	200.0	146.7
	185.0	186.7	143.3	110.0
	76.7	83.3	70.0	53.3
Average	**165.7**	**164.7**	**127.7**	**92.7**
Ratio of transpiration	**100.6%**	**100%**	**77.5%**	**56.3%**

4.2.2. Sampling for glucosinolate analyses in mature plants

In order to investigate the impact of drought stress on plants physiological state and secondary metabolism, especially on GS accumulation, sound GS determinations in roots and shoots of horseradish plants were required for the elucidation of both physiological and applicable aspects of GS accumulation in response to abiotic stress. The main question arises which plant organ should be analyzed and at which state? Horseradish roots represent physiological sinks for GS, and they are the commercial parts of the plant. However, leaves

are thought to be the organ in which GS are synthesized and they also contain considerable GS contents. However, leaves of various ages reveal different amounts of GS. Therefore, all leaves (various ages) of the same plant were analyzed for their total GS concentrations.

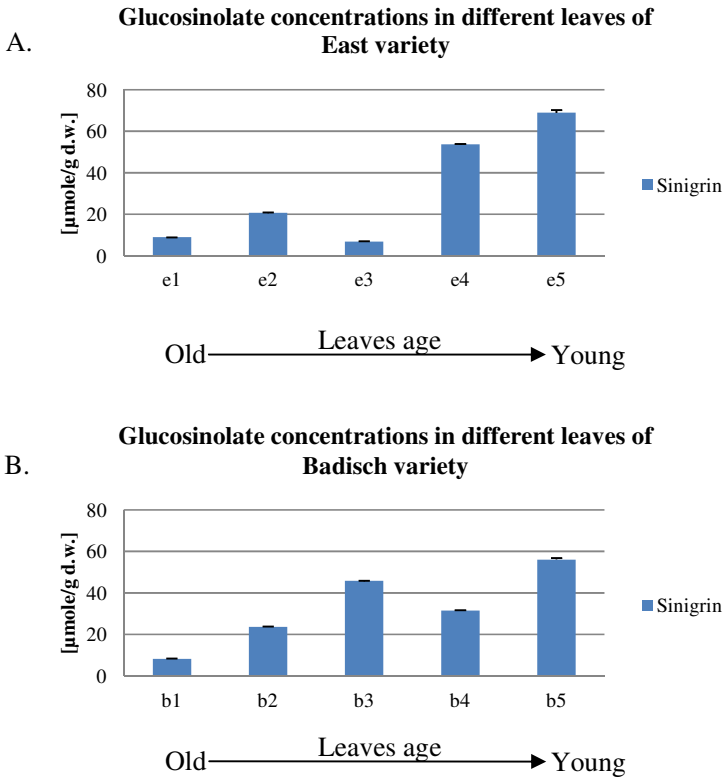


Figure 49: SI concentrations in different leaves of the same plant from (A) East variety and (B) Badisch variety. Data are average of double estimations. Bars resemble StD.

The GS concentrations of different leaves strongly differ. Young leaves reveal much higher GS concentrations and this finding is in accordance to previous reports (Brown et al., 2003). If we assume that all leaves behave similarly with respect of GS accumulation, we could deduce that the GS concentrations decline from about 60 to 10 µmole/g d.w (Figure 49). These differences could be due to differences in the rate of biosynthesis in old and young leaves and/or to translocation processes from old leaves (source) to young leaves (sink). Until now, no direct evidence is provided for enhanced GS biosynthesis in young leaves, which could explain their high GS concentrations. Active translocation of GS is known to take place between source (old leaves) and sink organs (young leaves and roots) (Brown et al., 2003; Chen et al., 2001). However, when the total GS concentrations in various leaves over the time period of two weeks were determined, rapid decreases in total GS concentrations of all investigated leaves were observed. This observation was expected for old leaves (source

organ), but TGS concentration decreases in young leaves indicate that GS are being translocated to another sink organ which presumably is the roots. This assumption is further supported by the fact that no GN was found in leaves, but considerable amounts have been detected in root samples later on.

The situation becomes even more complex, if we consider that in mature horseradish plants the tuber represents an additional large sink, in which GS are thought to be translocated (Brown et al., 2003). Moreover, farmers observed that horseradish plants harvested after the beginning of winter reveal better quality. Obviously, at the end of the vegetative period, when plant senescence is induced, further GS are translocated in the roots. In order to proof this assumption, GS concentrations of leaves of one plant at different times at the end of the growing season had been analyzed for their GS concentrations.

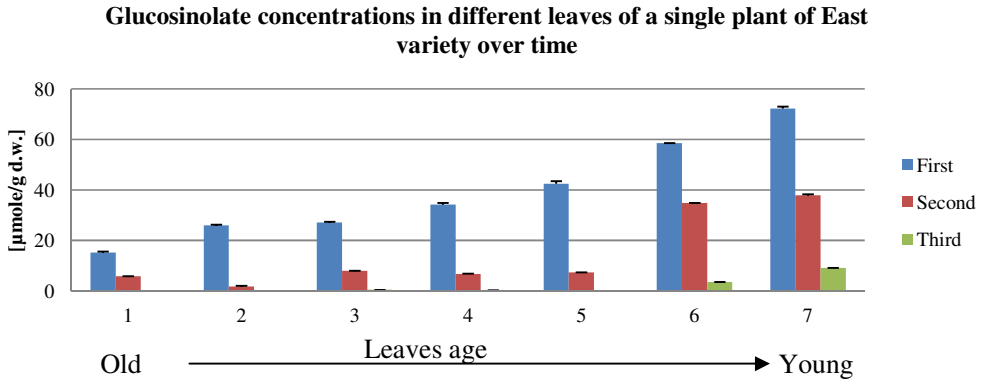


Figure 50: GS concentrations had been determined in leaf disks (≈ 2 cm in diameter), obtained by the use of a cork borer from the leaves of one plant, in one week intervals. First sampling was performed when all leaves were still vital and did not show any senescence. In the course of the experiment outer temperature decreased and after two weeks, most of the leaves had turned yellowish and showed typical senescence symptoms. Each value represents the average of duplicate determinations. Bars resemble StD.

At the end of season in the course of senescence GS concentrations decreased rapidly. Within two weeks, SI concentrations dropped from more than 70 µmole/g d.w. to less than 10 µmole/g d.w in the youngest leaf investigated, and SI was nearly completely lost from old leaves after two weeks (Figure 50).

The decrease in GS concentrations could be due to three possibilities: Degradation, remobilization and tanslocation. Degradation is very unlikely since there was no typical odor of mustard oils perceptible in the experimental field. Although not yet soundly documented to

occur in plants, remobilization could not be excluded. These data suggest that in the case of senescence, GS are efficiently translocacted from the leaves into the roots. However, the empirical findings of the farmers that at these stages of development the content of GS in the roots may strongly increase point to the fact that the GS are translocated into the roots (Courter and Rhodes, 1969). As consequence, the analysis of the impact of exogenous factors on GS concentrations was only performed for the roots, at a time when all leaves had been senescing. As major and secondary roots of horseradish contain different GS concentrations (Li and Kushad, 2004), representative mixed roots samples were taken, in order to obtain a sound analysis.

After having optimized the experimental procedure, the effects of drought stress on GS accumulation in horseradish mature plants of different varieties can be investigated.

4.2.3. Impact of drought stress on glucosinolate accumulation

Horseradish plants from different varieties were used to investigate water stress, especially drought, effects on GS accumulation. These different varieties represent some of the major horseradish varieties used in commercial plantations in Germany. Thus, results obtained also could be generalized on GS accumulation in horseradish plants in commercial plantations challenged by drought.

4.2.3.1. Effects of the drought stress on the plant growth of different varieties

Horseradish plants were subjected to various watering treatments in order to induce drought stress (see 4.2.1). Beside well watered control conditions, also an approach with excess water application was performed in order to address the question if other factors, e.g., anoxia, could also have an impact.

One major effect of stress is the inhibition of plant primary metabolism. Therefore, the effects of the treatments on the vegetative growth of the roots of horseradish plants from the three varieties were determined. The horseradish plants from the different varieties responded similarly. These results are summarized in (Table 16, A-C). As expected, drought stressed plants revealed less biomass production.

Table 16: Roots weight before and after growth in response to different treatments of East (A), Badisch (B) and Kröner (C) variety. Data correspond to the average of ten plants.

A.

Treatment East variety	Roots total weight/plant (g)			
	Sets weight (g)	StD	Roots weight after growth (g)	StD
Excess of water	24.2	4.1	88.3	11.7
Control	20.0	4.4	88.3	17.9
Moderate drought stress	24.4	7.4	73.1	14.6
Severe drought stress	25.9	5.9	73.1	9.5

B.

Treatment Badisch variety	Roots weight (g)			
	Sets weight (g)	StD	Roots weight after growth (g)	StD
Excess of water	28.29	6.27	93.72	16.88
Control	28.58	5.48	90.63	15.14
Moderate drought stress	26.71	5.09	67.61	7.95
Severe drought stress	29.30	5.58	76.6	4.34

C.

Treatment Kröner variety	Roots weight (g)			
	Sets weight (g)	StD	Roots weight after growth (g)	StD
Excess of water	30.71	13.57	86.4	30.97
Control	38.32	21.01	116.21	34.24
Moderate drought stress	30.36	11.4	73.37	19.09
Severe drought stress	36.32	19.50	88.81	29.89

Figure 51: Comparison of water treatments effect on the vegetative growth of control plants (left) and plants suffering from drought stress (right) after four months of treatment.

Generally, the WE plants developed similar root weights like the control plants. However, it is astonishing that the stressed plants revealed only small differences in the root weight, although their vegetative growth is highly reduced compared to that of the control plants (Table 16 and Figure 51). However, we have to consider that the main share of the root

weight of horseradish is due to the large storage root which is not involved in the water uptake.

Plants tolerance and demand of water is reported to vary in cultivars of the same species, or even in plants from the same cultivar adapted to different conditions (Morgan, 1984), which explains the observed slight differences in vegetative growth of plants from various varieties in response to different treatments. Upon severe drought, it is discussed that plants need to search for more water (Blum, 2011), which might cause the development of large roots in drought stressed plants

Decreases in root weight (yield) are a well known negative effect of drought stress. However, crop quality is not only determined by the amount of biomass produced, but also by the content of the relevant natural products, i.e., GS, which mainly affect the quality of horseradish tubers. Therefore, the elucidation of the effects of water stress (drought & water excess) on GS accumulation –apart from the scientific significance- also is important with respect to commercial aspects.

4.2.3.2. Impact of drought stress on glucosinolate accumulation

GS concentrations were determined in horseradish roots to assess the effect of water stress on GS accumulation. As outlined, the effect of drought stress on GS accumulation is also of major interest, since the GS concentration is crucial for the quality of horseradish roots.

The three varieties showed similar patterns for SI, GN and total GS in response to drought stress (Figure 52). Drought caused an increase in GS accumulation in roots of MS & SS plants compared to the control plants. However, the extent of GS increase was different among plants belonging to various varieties. As the various treatments resulted only in slight differences in dry matter accumulation, GS concentrations in horseradish roots on dry weight basis would not reveal different results. The corresponding data on dry weight basis are provided in the appendix (Figure A9).

The most effective treatment for increasing GS was MS, since this treatment caused a significant increase in all varieties; MS was also the only treatment that caused a significant difference according to the student T test (at 0.05 significance degree) in SI, GN and total GS concentrations (Figure 52, A-C).

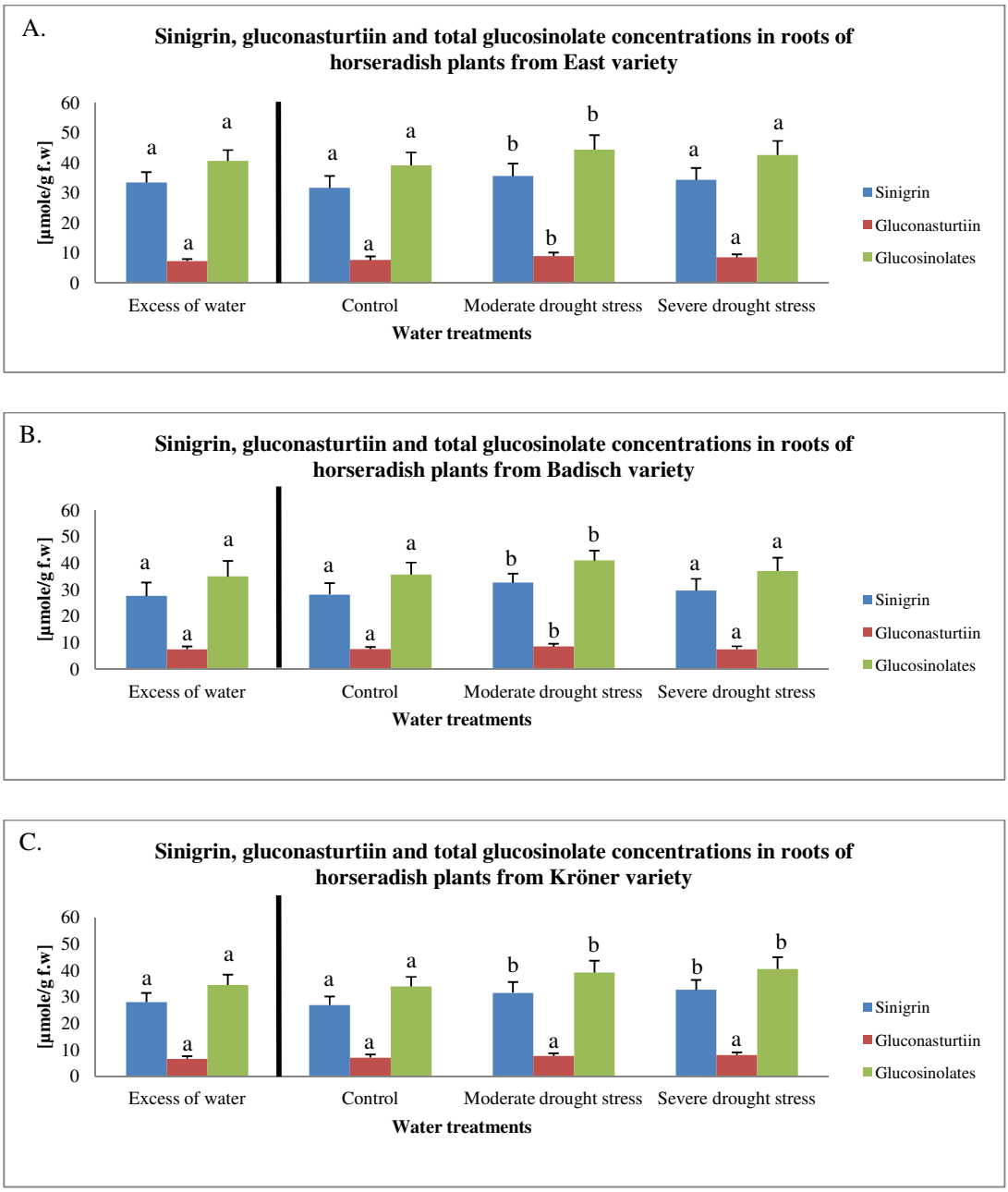


Figure 52: Effect of various treatments on SI, GN and total GS concentrations on fresh weight basis in horseradish roots of plants from East (A), Badisch (C) and Kröner (E) variety, respectively. Data are the average of douplicates for ten individual plants. Bars resemble StD. Different letters indicate significant differences compared to C plants, when data were analyzed according to student T test at 0.05 degrees of confidence.

Although WE and SS caused an increase in GS concentrations in almost all varieties, the increase caused by these treatments was not significantly different, except for the SS effect on SI and total GS concentrations in Kröner variety (Figure 52, C). When the control plants are compared with the plants grown under excess of water, it turned out that there are no

significant differences in the total GS concentrations. From this it can be deduced that the metabolic situation in both approaches should be similar and no additional stress, e.g., anoxia, was manifested.

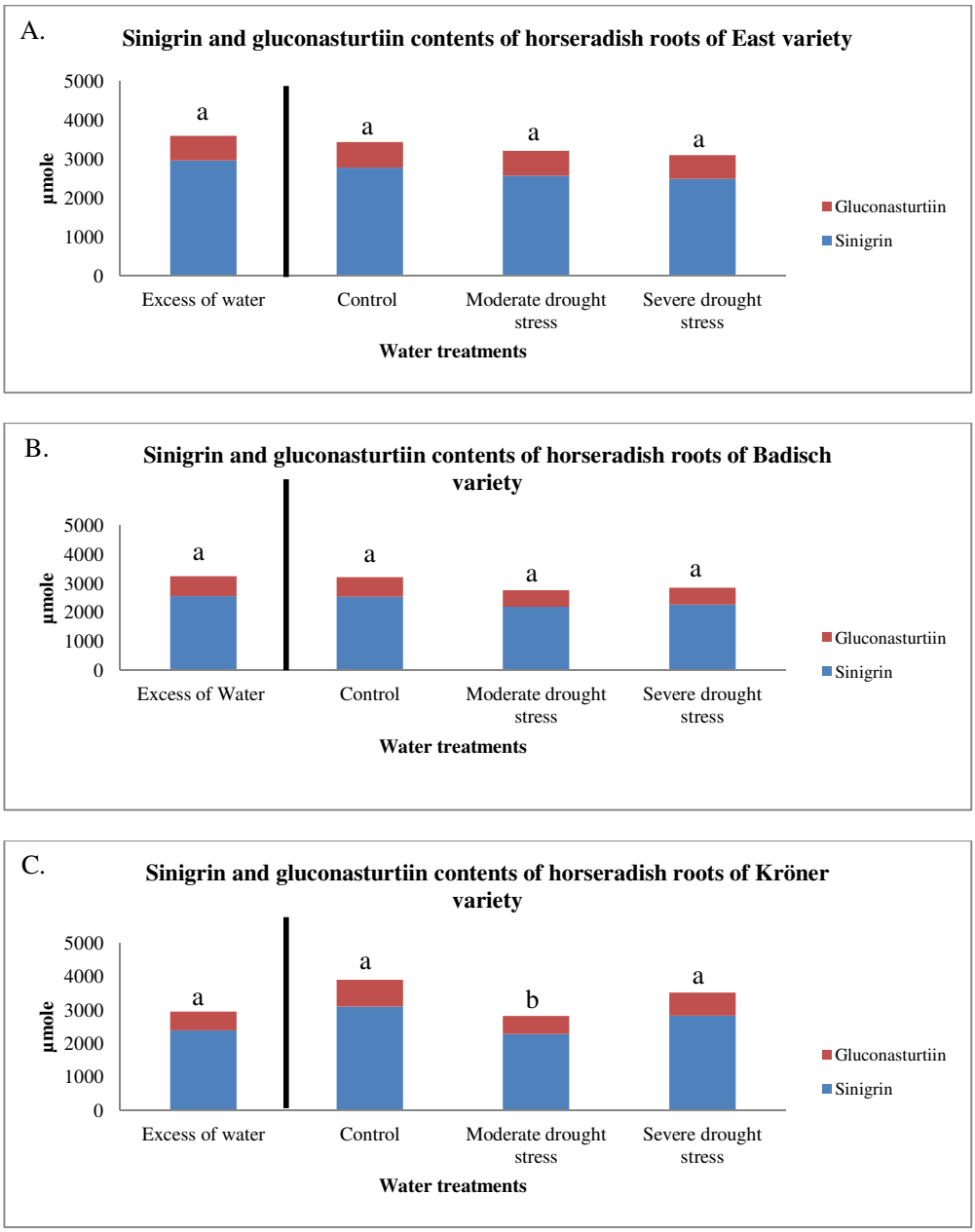


Figure 53: Total GS contents of roots of horseradish plants subjected to various treatments for East, Badisch and Kröner variety, respectively. Data are the average value of ten plants. Similar letters indicate insignificant differences among treatments, when data were analyzed according to student T test at 0.05 degrees of confidence.

An important issue for assessing the effect of abiotic stress on secondary metabolite production is the total content of secondary metabolites of the treated plants in comparison to that of the control plants. Although GS concentrations were enhanced in response to drought, produced horseradish tubers were smaller under these conditions. Therefore, the overall contents of GS have to be calculated.

The roots of the control plants contained always higher total GS than the roots of the drought stressed plants. However, due to the high biological variation, these differences were not significant on the base of the student T test at 0.05 degree of confidence (Figure 53).

The obtained results of total GS accumulation in roots of MS and SS plants are in agreement with previous reports concerning increased total GS accumulation in response to drought (Gershenzon, 1984; Jensen et al., 1996; Jing et al., 2010; Radovic et al., 2005). However, the extent of the drought effects varied among the used varieties and individual GS, which is also reflected in the scientific literature (Figure 52; Eslam, 2009; Moghadam et al., 2011). A corresponding effect of excess water on GS accumulation was also observed in *B. oleracea* (Khan et al., 2010). GS accumulation was different in various varieties, presumably due to the different tolerance of these plants to excess water supply.

Moderate stress affects GS accumulation more than severe stress, similar results have been obtained from *A. thaliana* and *B. carinata* plants subjected to drought (Jing et al., 2010; Schreiner et al., 2009). This was also observed for *B. campestris* plants, in which GS accumulation was increased in response to moderate NaCl (50 mM) but decreased upon treatment with higher NaCl concentrations (100 mM) (Keling and Zhujun, 2010). The results of the *in vitro* experiments also suggested the same (see chapter 4.1.7); GS concentrations were increased in response to moderate drought, simulated by 5% PEG (w/v). However, GS concentrations were decreased at severe drought stress, which was induced by higher PEG concentration.

Abiotic stresses in general and drought stress in particular are known to cause a decrease in vegetative growth. Hence, it is argued that the higher natural product concentrations observed in stressed plants are the result of metabolites concentration due to lesser biomass (Selmar and Kleinwächter, 2013, Gershenzon, 1984). The data obtained here, showed that GS concentrations are indeed increased in response to drought. However, this increase did not compensate for the huge reduction of vegetative growth in response to drought. Therefore,

although quality of horseradish tubers can be enhanced by using drought, negative effects on vegetative growth effect cannot be excluded.

To estimate how the different treatments affected the plant stress metabolism, GABA concentration as stress indicator has been determined. Evaluating the physiological state of the plants will aid in correlating it to GS accumulation.

4.2.3.3. Effects of drought on GABA accumulation

Accordingly, parallel to GS analysis, GABA concentrations were quantified. Indeed, the roots of plants of all treatments accumulated higher GABA concentrations than the control. However, high variations among plants and the various varieties were observed (Table 17). Due to the slight differences in dry matter accumulation, no major differences were observed between the GABA concentration pattern on dry weight and fresh weight basis (Table 17, A-C).

Table 17: GABA concentrations on dry and fresh weight basis in all three analyzed varieties (East, Badisch and Kröner, A-C). Data are duplicate estimations of two independent pooled samples.

A.

Treatment (East)	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Excess of water	120.4	11.5	42.0
Control	102.1	9.7	36.4
Moderate stress	123.3	24.2	43.6
Severe stress	121.2	22.3	43.6

B.

Treatment (Badisch)	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Excess of water	74.49	4.13	25.69
Control	67.89	1.05	23.97
Moderate stress	79.55	0.44	27.56
Severe stress	70.70	2.02	24.89

C.

Treatment (Kröner)	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Excess of water	64.28	3.07	22.69
Control	56.46	3.81	20.36
Moderate stress	63.07	2.82	22.8
Severe stress	55.95	3.26	20.86

Although it turned out that GABA does not represent a reliable marker for general stress situations in *in vitro* plants treated with ABA and NaCl (see 4.1.8 & 4.1.9), for the estimation of the general stress status of soil grown plants it represents a sound marker.

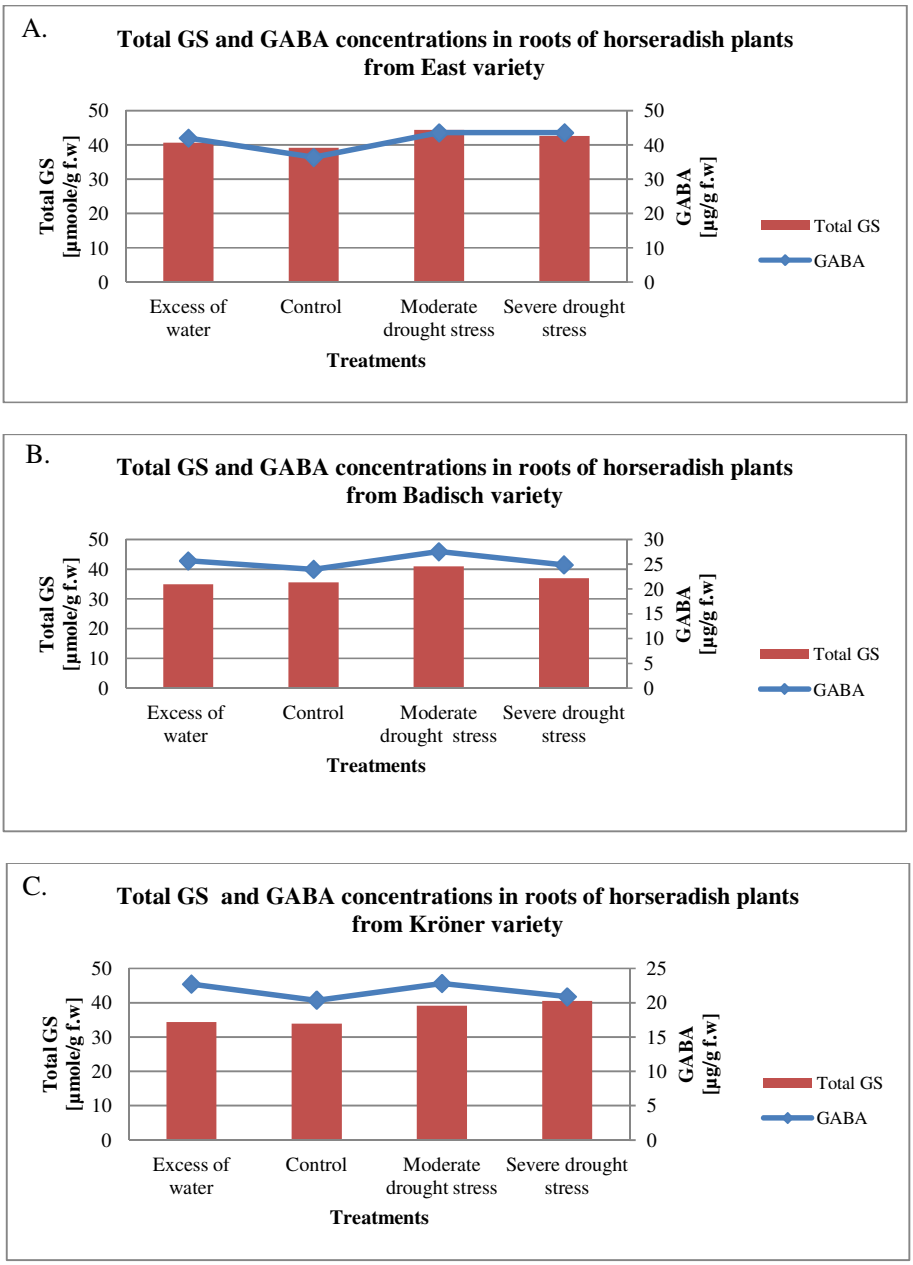


Figure 54: Total GS and GABA concentrations on fresh weight basis in roots of horseradish plants subjected to different water treatments in East (A), Badisch (B) and Kröner (C) varieties.

For improved analysis of GABA and total GS concentrations, both compounds were plotted in the same graph. Their concentrations on fresh weight basis were used to reflect the conditions of viable plants. Amazingly, both compounds followed similar patterns. Total GS

concentrations increased in case of moderate stress as indicated by an increase in GABA concentrations. Therefore, it can be concluded that changes in plant physiological state -caused by stress- lead to an enhanced GS accumulation as compared to the control plants (Figure 54).

GABA accumulation in response to water stress (excess water as well as drought) is well documented (Bown and Shelp, 1997; Shelp et al., 1999). However, different species and cultivars accumulate different amounts of GABA in response to abiotic stresses (Vasquez-Robinet et al., 2008; Saeedipour and Moradi, 2012). Moreover, slight differences in water needs might also have affected GABA accumulation in treated plants of different varieties (Table 17, A-C).

Similar patterns of GS and GABA accumulation indicate that both metabolites respond to drought stress in a similar manner, as also indicated in previous studies (Figure 54; Jensen et al., 1996; Selmar and Kleinwächter, 2013; Bown and Shelp, 1997). Although differences in their accumulation also exist, as discussed earlier (section 4.1.).

The results obtained so far, indicate the possibility of GS concentration modulation in horseradish in response to drought. However, with respect to putative commercial applications, this possibility is limited due to the negative effects of drought on vegetative growth. Therefore, signaling molecules that intervene with plant responses to stress were chosen for further studies. Signaling molecules, such as salicylic acid, may modulate GS accumulation, and can relatively easily be applied in the field. Therefore, the effect of salicylic acid on GS accumulation has been investigated.

4.2.4. Effects of salicylic acid treatments on glucosinolate accumulation in horseradish.

Phytohormones and signaling molecules are reported to have effects on GS accumulation (Mewis et al., 2005). With respect to putative commercial applications, salicylic acid (SA) was chosen to investigate exemplarily the effect of signaling molecules on GS accumulation in horseradish mature plants. It is well known that exogenously applied SA affects GS accumulation (Kiddle et al., 1994). SA plays an important role in plant responses to stress, and due to its interaction with jasmonic acid –among other factors- SA is responsible for regulating GS accumulation in plants (Mewis et al., 2006). In contrast to the general approach described in the last chapter, samples for GS analysis were taken from leaves and roots.

Table 18: Roots weight before and after growth of plants subjected to different SA treatments. Data are the average value of seven plants.

Treatment SA [mM]	Roots weight (g)			
	Sets weight (g)	StD	Roots weight after growth (g)	StD
0	24.55	6.33	68.10	12.79
1	19.30	7.23	52.12	10.52
5	19.7	3.83	58.70	15.17

In general, the control plants developed similar roots compared to the plants treated with SA, and the plants subjected to 5 mM SA developed slightly larger roots than plants treated with 1 mM SA (Table 18).

SI and total GS concentrations on dry as well as on fresh matter basis did not increase in response to SA treatments. SI, GN and total GS concentrations in roots of plants subjected to different treatments were very similar, resulting in no significant difference, when data were analyzed according to the student T test at 0.05 degree of confidence (Table 19).

Table 19: SI, GN and total GS accumulation in roots of horseradish plants subjected to different SA treatments. Data represent the average value of seven plants for each treatment, and duplicates of individual plants. Similar letters indicate statistical insignificance at 0.05 according to student T test.

Treatment SA [mM]	Glucosinolates [µmole/g d.w.]					
	Sinigrin	StD	Gluconasturtiin	StD	Total glucosinolates	StD
0	64.62a	11.80	18.62a	3.22	83.24a	13.57
1	61.72a	4.8	20.14a	2.66	81.87a	5.32
5	63.49a	10.42	17.97a	2.73	81.46a	8.47
Treatment SA [mM]	**Glucosinolates [µmole/g f.w.]**					
	Sinigrin	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
0	21.72a	3.67	6.25a	0.88	27.97a	4.00
1	21.64a	1.66	7.09a	1.14	28.73a	2.28
5	22.50a	4.78	6.37a	1.16	28.86a	4.71

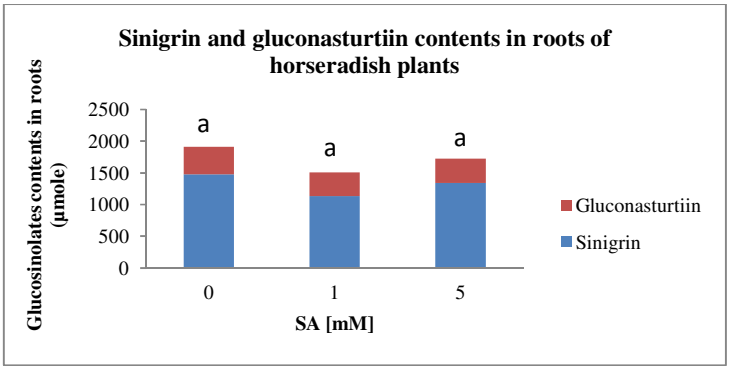


Figure 55: SI and GN contents in horseradish roots of horseradish plants subjected to different SA treatments. Data are average of seven plants for each treatment.

As outlined earlier, in addition to the GS concentration, changes in the total GS content also could contribute to elucidate the putative impact of exogenous factors on GS metabolism. However, the small increase in total GS concentrations did not compensate the difference in the biomass (Figure 55). GS content was the highest in the roots of the control plants and least in roots of plants subjected to 1 mM SA. However, no significant difference were found between the different treatments when data were analyzed according to student T test at 0.05 degree of confidence (Figure 55).

Table 20: GABA concentrations in horseradish roots of plants subjected to different treatments. Data presented are the average of double estimations of two independent pooled samples.

Treatment SA [mM]	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
0	35.67	1.63	12.0
1	34.56	1.66	12.1
5	42.98	0.30	15.06

In order to estimate the stress status, also in this approach, GABA concentrations were analyzed. GABA concentrations were found to increase in the plants treated with 5 mM SA compared to the control plants, however, no significant differences between the control and the 1 mM SA treatment could be detected (Table 20). This indicates that only in plants treated with 5 mM SA stress reactions are induced. The patterns of total GS and GABA accumulation become obvious when their concentrations are plotted together in one graph. Concentrations on fresh weight basis were used to resemble the status of viable plants (Figure 56).

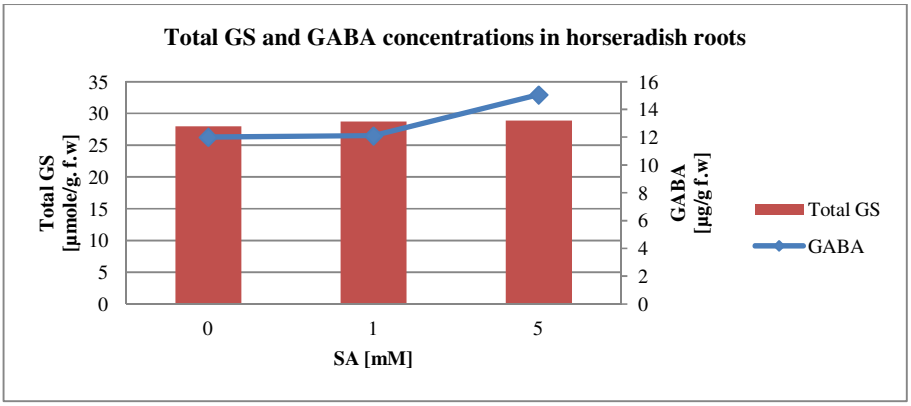


Figure 56: Total GS and GABA concentrations on fresh weight basis in roots of horseradish plants treated with 0.0, 1.0 and 5.0 mM SA, respectively.

These data are in contradiction to the literature, which reported that GS accumulation increases in SA treated plants (Kiddle et al., 1994). However, these authors analyzed the leaves. Thus, in contrast to the other experiments described earlier, in this approach the experiment was terminated far before the vegetative period ended and thus also the leaves were analyzed.

Table 21: SI and total GS accumulation in horseradish leaves subjected to different SA treatments on dry and fresh matter basis. Data are average of duplicate determinations of seven plants. Data were statistically insignificant at 0.05 degree of confidence according to student T test, as indicated by similar letters.

Treatment SA [mM]	Glucosinolates [µmole/g d.w.]			
	Sinigrin	StD	Total glucosinolates	StD
0	33.21a	7.1	33.21a	7.1
1	31.32a	6.29	31.32a	6.29
5	39.64a	13.10	39.64a	13.10
Treatment SA [mM]	**Glucosinolates [µmole/g f.w.]**			
	Sinigrin	**StD**	**Total glucosinolates**	**StD**
0	4.73a	1.2	4.73a	1.2
1	4.43a	0.41	4.43a	0.41
5	5.32a	0.17	5.32a	0.17

As already shown for *in vitro* plants, in leaves only SI was detected; therefore, SI corresponds to total GS in the leaves. Leaves of plants treated with 5 mM SA accumulated higher total GS concentrations as compared to the leaves of the control plants, on dry as well as fresh weight basis (Table 21). In contrast, leaves of plants treated with 1 mM SA showed a decrease in total GS concentrations.

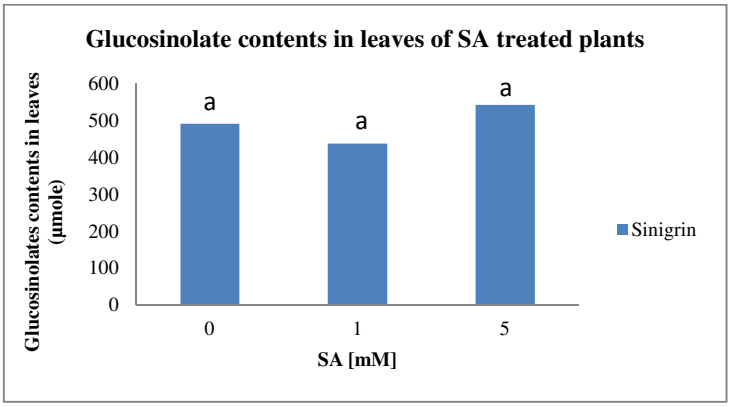


Figure 57: Total GS contents in horseradish leaves upon SA application to plant roots. Differences in total GS contents were insignificant according to student T test at 0.05 degree of confidence as indicated by similar letters. Data points represent the average content of seven plants.

The total GS concentrations were increased in the leaves of SA treated plants. However, for a solid evaluation of the effects of various treatments on GS accumulation, total GS contents have to be calculated. Only higher total GS contents in SA treated plants can prove a real increase in GS biosynthesis in the treated plants. Indeed, total GS content was higher in leaves treated with 5 mM SA, but decreased in the leaves subjected to 1 mM SA compared to the leaves of control plants. However, these differences were statistically insignificant when analyzed according to student T test at 0.05 degree of confidence (Figure 57). Thus, solid conclusions on GS accumulation cannot be drawn.

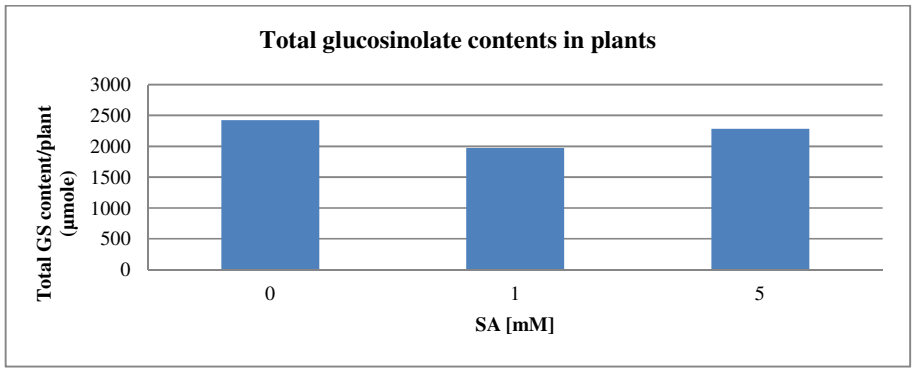


Figure 58: Total GS contents in horseradish plants subjected to various concentrations of exogenous SA.

To present a general view, total GS contents of the plants were calculated. The obtained data show that total GS contents of the control plants were similar with those treated with 5 mM SA. However, plants subjected to 1 mM SA contain slightly less total GS (Figure 58). These data show that no increase in GS biosynthesis could be deduced, and that the obtained data might be influenced by differences in biomass. However, another issue was observed; plants subjected to 5 mM SA reveal enhanced accumulation of GS. However, no corresponding increase in total GS concentrations in the roots is observed. This indicates that no or only little enhancement of GS translocation from leaves to roots takes place in these plants. Therefore, GS might be translocated from leaves to roots in response to certain physiological processes, such as the previously mentioned leaf senescence. Alternatively, the specific accumulation of GS in the leaves might provide certain benefit for the plant. The actual cause for this observation awaits more investigation.

Discussion:

Minor effects of exogenously applied SA on vegetative growth of roots was also previously reported for *B. oleracea* (Van Dam et al., 2003). However, only slight differences in total GS concentrations were observed in roots of treated horseradish plants. Moreover, the species used, environmental conditions and application organs were found to affect GS accumulation in plants treated with SA (Redovnikovic et al., 2008a; Van dam et al., 2003). On the other hand, the observed effects of high SA application (5 mM) on GS concentration in *A. rusticana* leaves are in agreement with results reported earlier for *B. napus* and *B. olerace* (Kiddle et al., 1994; Van Dam et al., 2003). Moreover, corresponding decrease in the accumulation of GS in response to low SA concentrations was also noticed in *B. nigra* plants (Table 21; Van Dam et al., 2003).

The role of SA in GS accumulation is not clear. Generally, low SA concentrations resulted in minor effects on GS accumulation. However, at high concentrations, SA results in corresponding increase in GS accumulation (Van Dam et al., 2003; Ludwig-Müller et al., 1997; Kiddle et al., 1994), possibly through synergetic effects of different stress signaling pathways.

For example, both SA and JA are known to regulate GS synthesis, and SA was proposed to decrease GS concentrations through suppression of JA signaling pathway (Mewis et al., 2005). However, SA has an important role in plant resistance towards pathogens, and its role in plant resistance to insects is emerging (Mikkelsen et al., 2003). Under certain stresses, SA was found to stimulate resistance instead of antagonizing defenses induced by JA. In plants JA, ET and SA signaling pathways can act synergistically or antagonistically (Mewis et al., 2006). Therefore, manifestation of SA responses depends on used concentrations, treated organ and species used. These factors affect plants response-in terms of GS synthesis- to exogenous SA application (Van Dam et al., 2003; Ludwig-Müller et al., 1997).

Concerning GABA, SA is known of activating genes related with plant responses to stress (Mewis et al., 2006), and GABA accumulation is sensitive to a wide variety of biotic and abiotic stresses. Therefore, it is highly probable that the GABA concentration increases in response to stress signals caused by SA application (Shelp et al., 1999). An enhancement of the GABA concentration in the roots indicates that plants are suffering from stress (Bouche

and Fromm, 2004), although this stress did not result in GS accumulation in roots. Therefore, GS concentrations were investigated in leaves.

The experiments conducted on *in vitro* horseradish plants and horseradish mature plants grown under rain shelter confirmed the possibility of enhancing total GS concentrations in horseradish tubers. Drought and salinity stress were effective in total GS concentration enhancement. However, horseradish is grown commercially in the field. So any attempt to improve horseradish tubers quality should be conducted in the field. Therefore, the effects of abiotic factors effects were also investigated in plants grown in the field.

4.3. Effects of drought and salinity on glucosinolate accumulation in roots of horseradish plants grown in field

In addition to the model experiments in the rain shelter, field experiments were conducted in order to investigate the possibility to modulate GS concentrations by applying moderate stress under practical conditions. Thus, the deliberate application of abiotic stresses to improve horseradish tubers quality for commercial production can be validated under field conditions.

In order to induce differences in water supply, horseradish plants were grown in two designs; furrows (F) enabled water harvesting, and this represented well watered conditions. On the other hand, ridges (R), reducing the amount of provided water. As mentioned, the soil design caused differences in water absorption: In this experiment, water content in hills was in average only 65% of the water content in furrows, when water content of all predetermined locations was calculated. In order to induce salt stress, various plants cultivated under these two watering regimes were also treated with NaCl; four different treatments (0, 25, 50 and 100 mM NaCl) had been used. Correspondingly, initial letters “F” or “R” were used to indicate water treatments and numbers were used to indicate NaCl treatment, e.g., 25, stands for plants receiving 25 mM of NaCl.

For investigating drought and salinity effects on horseradish plants cultivated in the field, plants from the three varieties were randomly distributed, and subjected to treatments mentioned earlier.

4.3.1. Effects of drought and salinity on glucosinolate accumulation in roots of horseradish plants from various varieties grown in field

To investigate drought and salinity effects on horseradish plants originating from various varieties, similar sets were used. At the end of the cultivation period, plant roots were harvested to determine their vegetative growth. Generally, plants grown in ridges reveal considerable higher roots weights as compared to the plants cultivated in furrows (Table 22), and the observed pattern was not changed by NaCl treatments (Table 23, A-C).

In contrast to general pattern of vegetative growth, the opposite was obtained in plants from the East variety in response to 50 mM NaCl treatments (Table 23, A). Most probably this pattern was caused by some material loss during tedious practices of excavating plants.

Table 22: Roots weight of plants subjected to differential watering due to soil design from various varieties.

Variety	Soil design	NaCl [mM]	Sets weight (g)	StD	Root weight after growth (g)	StD
East	Furrows	0	26.08	6.91	176.57	103.41
	Ridges	0	20.22	7.0	292.86	267.67
Badisch	Furrows	0	27.05	4.23	182.86	84.77
	Ridges	0	26.94	3.79	287.71	152.89
Kröner	Furrow	0	31.42	10.74	306.86	256.02
	Ridges	0	37.54	13.45	438.00	199.10

Table 23: Roots weight before and after growth of plants subjected to different treatments for East (A), Badisch (B) and Kröner (C) horseradish varieties. Data are the average of seven plants. Highlighted boxes resemble data already presented in Table 22.

A.

Soil design	NaCl [mM]	Sets weight (g)	StD	Roots weight after growth (g)	StD
Furrows	0	26.08	6.91	176.57	103.41
Ridges	0	20.22	7.0	292.86	267.67
Furrows	25	24.43	5.46	175.67	191.16
Ridges	25	22.34	6.2	334.8	247.05
Furrows	50	22.19	3.54	362.28	472.14
Ridges	50	21.45	3.88	172.29	201.45
Furrows	100	20.76	2.86	164.86	92.85
Ridges	100	21.88	5.89	366.57	299.60

B.

Soil design	NaCl [mM]	Sets weight (g)	StD	Roots weight after growth (g)	StD
Furrows	0	27.05	4.23	182.86	84.77
Ridges	0	26.94	3.79	287.71	152.89
Furrows	25	27.15	4.77	219	101.76
Ridges	25	27.11	3.78	278.86	148.99
Furrows	50	23.45	3.48	248.92	139.91
Ridges	50	25.64	4.78	564.40	297.86
Furrows	100	28.13	2.08	278.42	114.14
Ridges	100	25.38	3.33	405.43	189.84

C.

Soil design	NaCl [mM]	Sets weight (g)	StD	Roots weight after growth (g)	StD
Furrows	0	31.42	10.74	306.86	256.02
Ridges	0	37.54	13.45	438.00	199.10
Furrows	25	28.48	10.78	310.8	195.93
Ridges	25	39.99	21.91	594.33	408.54
Furrows	50	37.85	14.11	360.86	164.02
Ridges	50	30.06	11.92	462.86	281.88
Furrows	100	31.22	16.36	265.14	146.65
Ridges	100	31.06	11.06	463.43	337.45

In order to evaluate the effects of various treatments on GS accumulation, total GS, SI and GN concentrations were investigated. Generally, the plants grown in furrows revealed higher total GS concentrations than the plants grown in ridges (Table 24). GS patterns of accumulation were slightly affected due to differences in dry matter accumulation. For simplicity, SI, GN and total GS concentrations will be presented on fresh matter basis. Data for GS concentrations on dry matter basis is provided in the appendix (Table A3).

Table 24: SI, GN and total GS concentrations in roots of horseradish plants from various varieties subjected to different watering regimes. Similar letter indicate significantly indifferent results when data were analyzed according to student T test at 0.05 degree of confidence.

			Glucosinolates [µmole/g f.w.]					
Variety	**Soil design**	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
East	**Furrows**	**0**	31.50	6.42	4.93	0.56	36.43a	5.88
	Ridges	**0**	29.52	4.19	4.56	0.97	34.08a	3.71
Badisch	**Furrows**	**0**	31.07	5.12	3.96	1.24	35.03a	5.25
	Ridges	**0**	28.38	5.86	4.04	0.76	32.41a	5.98
Kröner	**Furrows**	**0**	35.20	6.69	4.03	0.73	39.23a	6.22
	Ridges	**0**	30.55	4.62	3.39	0.46	33.94a	4.69

The data obtained show opposite pattern to that expected for GS accumulation, i.e., higher GS concentrations in the plants cultivated in ridges due to drought. However, GS concentrations were decreased in the plants grown in ridges (Table 24). It is expected that due to the humid summer, soil design resulted in normal water content for plants grown in ridges and adverse conditions due to excess of water content in furrows. Obviously, plants grown in furrows suffered from excess water, e.g., partial anoxia. Also in the salt experiment, plants grown in furrows revealed higher concentrations compared to plants cultivated in ridges (Table 25, A-C). This supports our previous assumption that ridges reveal normal contents of water, while furrows provided the plants with excess of water amounts than the plants needed. This argument also explains the observed results concerning the vegetative growth (Table 25, A-C).

Table 25: SI, GN and total GS concentrations in roots of horseradish plants subjected to different treatments on fresh weight basis. Differences in SI, GN and total GS concentrations were not statistically significant at 0.05 degree of confidence according to student T test. Similar letter indicate significantly indifferent results. Data are average of double estimations of seven plants. Highlighted data have already been presented in Table 24.

A.

East variety		Glucosinolates [µmole/g f.w.]					
Soil design	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
Furrows	**0**	31.50	6.42	4.93	0.56	36.43a	5.88
Ridges	**0**	29.52	4.19	4.56	0.97	34.08a	3.71
Furrows	**25**	31.58	4.68	4.27	0.59	35.85a	4.41
Ridges	**25**	32.33	3.83	3.99	0.78	36.32a	3.26
Furrows	**50**	31.94	5.34	4.74	0.42	36.68a	4.34
Ridges	**50**	27.94	5.28	4.82	1.03	32.75a	5.57
Furrows	**100**	33.28	6.49	4.49	1.00	37.77a	5.89
Ridges	**100**	29.78	3.89	4.82	1.40	34.60a	3.04

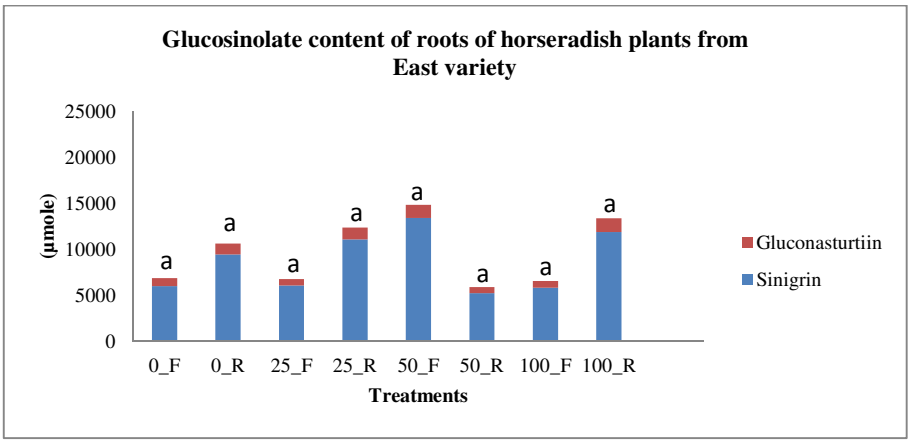
B.

Badisch variety		Glucosinolates [µmole/g f.w.]					
Soil design	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
Furrows	**0**	31.07	5.12	3.96	1.24	35.03a	5.25
Ridges	**0**	28.38	5.86	4.04	0.76	32.41a	5.98
Furrows	**25**	30.78	3.65	3.48	0.61	34.25a	3.54
Ridges	**25**	27.72	5.42	3.94	0.69	31.66a	5.14
Furrows	**50**	31.27	5.32	4.17	1.21	35.43a	4.50
Ridges	**50**	30.60	1.27	3.95	0.75	34.55a	1.82
Furrows	**100**	30.66	4.65	4.25	0.57	34.91a	4.69
Ridges	**100**	29.18	1.70	3.71	0.48	32.89a	1.77

C.

Kröner variety		Glucosinolates [µmole/g f.w.]					
Soil design	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
Furrows	**0**	35.20	6.69	4.03	0.73	39.23a	6.22
Ridges	**0**	30.55	4.62	3.39	0.46	33.94a	4.69
Furrows	**25**	34.32	2.68	3.75	0.51	38.07a	2.81
Ridges	**25**	28.65	3.19	3.32	0.71	31.97b	2.80
Furrows	**50**	36.17	3.57	3.67	0.56	39.83a	3.52
Ridges	**50**	29.90	4.30	3.67	0.87	33.57b	4.81
Furrows	**100**	30.85	5.41	3.70	0.59	34.55a	4.88
Ridges	**100**	31.23	5.70	4.10	0.63	35.32a	5.24

A.



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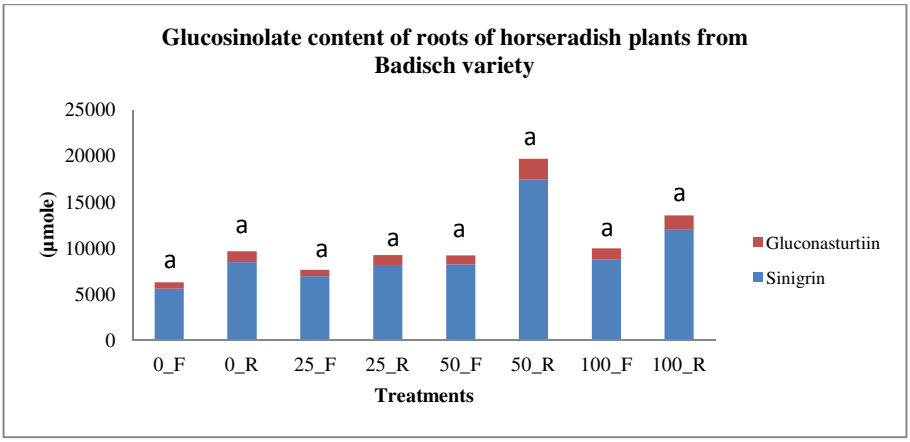

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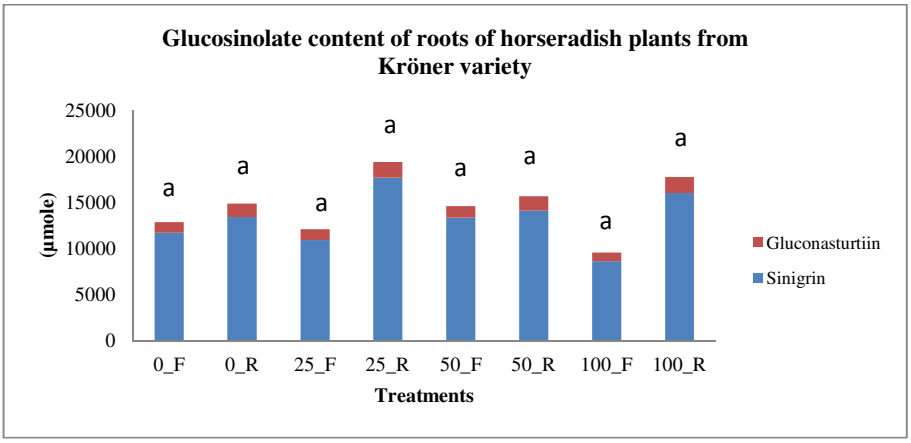


Figure 59: SI and GN (total GS) contents in roots of horseradish plants subjected to different treatments. Data are average of seven plants. Similar letters indicate no significant differences among treatments of same NaCl concentration.

Data obtained show no effect of salt on GS concentration. Therefore, in order to verify if the treatments caused differences in total GS accumulation in the plants, total GS contents were calculated. Total GS contents are a crucial aspect to investigate the effects of treatments on vegetative growth and total GS concentration. Again total GS contents did not reveal a clear

pattern for GS accumulation in response to salt treatments. In contrast, due to the huge differences in vegetative growth, plants cultivated in ridges revealed higher total GS contents compared to plants cultivated in furrows (Figure 59, A-C).

Huge differences were observed in total GS contents between plants cultivated in furrows and ridges soil designs. However, these differences were not statistically significant between plants treated with the same NaCl solutions at 0.05 confidence degree, when analyzed according to student T test (Figure 59, A-C).

The manifestation of stress in the plants subjected to various treatments was investigated by determining GABA concentrations. However, GABA accumulated differently in various varieties. Therefore, no clear pattern of GABA accumulation in plants cultivated in the field was obtained, possibly due to the sensitivity of GABA to be accumulated in the course of a wide variety of stresses, usually prevailing in the field. Detailed data concerning GABA accumulation on dry and fresh weight basis are presented in the appendix (Table A4).

Discussion

In general, large root system may aid plants receiving low water supplement to enhance water absorption under drought conditions. However, storage roots are not included in water absorbance. Accordingly, deeper root systems in drought stressed plants have been observed (Blum, 2011). On the other hand, the hilling up made soils in ridges less compact compared to soils in furrows. Loosened soils are favorable for the growth of horseradish plants. Compacted and not well aerated soils are not favorable for horseradish growth, which might cause the decrease in the roots weight observed for the plants cultivated in furrows (Courter and Rhodes, 1969). Moreover, high soil water contents cause decreases in yield, e.g., excess water causes 25-30% decreases in corn yield annually (Qiu et al., 2007). It is assumed that due to the humid summer, plants grown in ridges received enough water for good growth. On the other hand, the furrow design provided the plants with water amounts higher than their optimal needs, causing retardation in growth.

NaCl affected roots weight of plants grown in ridges. NaCl treatment at the colar region may have increased osmotic stress locally, causing enhanced growth of the root system to deeper moist soils lacking NaCl. Similar effects have been reported for plants challenged by partial drought, which developed deeper root systems (Blum, 2011).

Nonetheless, drought and NaCl are known to affect total GS accumulation under controlled conditions (Keling and Zhujun, 2010; Jing et al., 2010). In this experiment, water treatments seem to be more effective in determining total GS concentrations than NaCl treatments, and the increase in total GS increased in plants grown in furrows indicates that the plants may suffer from soil compaction, bad aeration and probably high water contents (Courter and Rhodes, 1968). Excess of water supply was found to have different effects on GS concentration in *B. oleraceae,* presumably due to the amount of added water (Khan et al., 2010; Khan et al., 2011). Although, total GS accumulation might be most affected by the high vegetative growth of the plants cultivated in ridges (Table 23; Gershenzon, 1984; Selmar and Kleinwächter, 2013).

The effects of various treatments on stress metabolism were evaluated by analyzing GABA concentrations as reliable stress indicator. GABA tends to accumulate rapidly in response to a wide variety of biotic and abiotic stresses (Shelp et al., 1999; Bown and Shelp, 1997). Moreover, GABA concentrations are determined by metabolism, catabolism and transport within the plant (Shelp et al., 1999). Therefore, interactions between different stresses usually prevailing in the field might affect plants in a different manner compared to individual stresses induced in closed systems (Obata and Fernie, 2012). These coherences might result in the observed fluctuations of the GABA concentrations.

4.3.2. Effects of drought on glucosinolate accumulation in roots of horseradish plants from Badisch and Kröner variety cultivated in the field in 2011.

To verify the results obtained, the field experiments were repeated. However, only the different water treatments had been investigated. Two varieties, i.e., Badisch (B) and Kröner (K) were grown in ridges and furrows soil design to control the water supply, i.e., plants grown in ridges (R) should receive less water than plants grown in furrows (F).

Table 26: Weighs of roots of horseradish plants from Badisch (B) and Kröner (K) variety subjected to different treatments (after growth period). Data are the average of 25 plants from each variety.

Variety	Soil Design	Roots weight after growth
Badisch	Furrows	170.00
Badisch	Ridges	343.44
Kröner	Furrows	149.62
Kröner	Ridges	256.67

As shown in the previous year, plants grown in ridges were much larger, and the biomass of their roots was markedly enhanced (Table 26). Unfortunately, also the summer of 2011 was very rainy, too. Accordingly, the soil of the ridges does not represent water limited conditions to induce drought stress. The difference in growth can be explained by the fact that the loosened, well aerated soil in ridges is more favorable for the growth of horseradish plants as compared to the compact soils in furrows (Courter and Rhodes, 1968), and this might cause the observed difference in root growth of the plants cultivated in the two soil designs. Moreover, it has been reported that even slight or partial drought positively affects the root growth (Blum, 2011).

Also the results concerning GS fully confirm those from the previous year: GS concentrations in plants grown in the ridges are lower than those of the plants cultivated in furrows (Figure 60). GS concentrations on dry weight basis are provided in the appendix (Table A5).

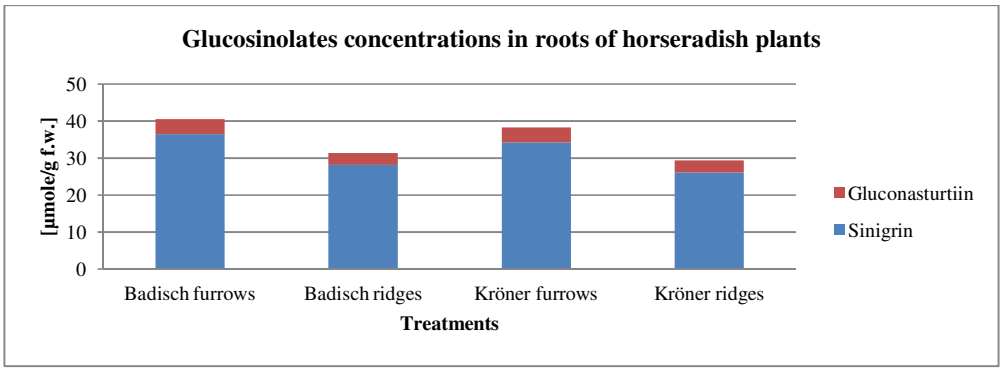


Figure 60: SI and GN (Total GS) concentrations in plants from Badisch (B) and Kröner variety on fresh weight basis. Data are average of duplicates.

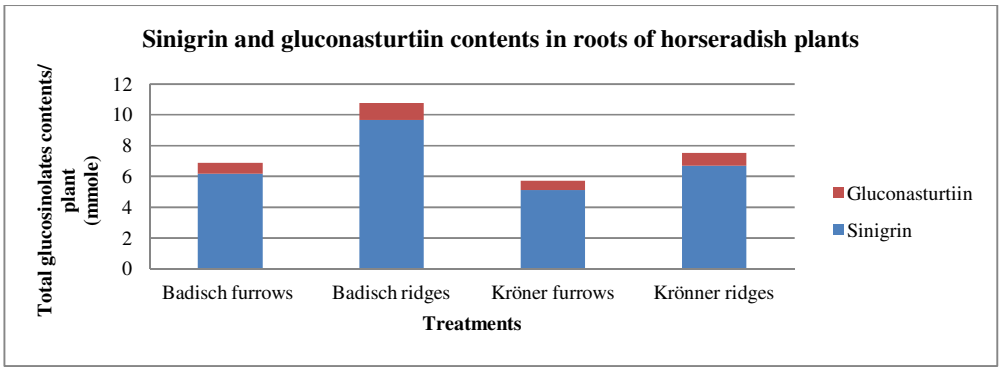


Figure 61: SI and GN (Total GS) contents of roots of Badisch and Kröner horseradish plants grown in furrows and ridges.

Although total GS concentrations were increased by 30% in furrows plants, such high increase could not overcome 100% and 70% increase in roots weight of plants grown in ridges compared to the plants cultured in furrows. Due to such high differences in the vegetative growth, total GS contents were 56 and 32% higher in ridges plants of Badisch and Kröner varieties, respectively (Figure 61).

As in the previous year, GABA concentrations were also accumulated differently in roots of plants from the two varieties in response to different treatments. Detailed data for GABA accumulation on dry and fresh weight basis are provided in the appendix (Table A6).

GS were shown to accumulate in response to adverse conditions (Selmar and Kleinwächter, 2013). Unfavorable growth conditions for horseradish plants, such as excess water supply and compacted soils of the furrow design, seem to cause plant stress (Courter and Rhodes, 1969; Khan et al., 2010).

GABA accumulates in response to wide array of stresses (Shelp et al., 1997), and any interference with plants might change observed results. Moreover, plants from different species and cultivars showed altered GABA accumulation response (Saeedipour and Moradi, 2012).

Effects of exogenous factors on GS accumulation in horseradish plants cultivated in the field were not expected. Different soil designs caused differences in the water contents of the soils of the two designs. However, it seems to be very likely that the reduction in absorbed water did not induce drought in plants cultivated in ridges, possibly due to humid summer in Germany. It is suggested that the plants cultivated in furrow soil design suffered from excess water supply, which adversely affects plants growth and causes an increase in GS concentration. On the other hand, plants cultivated in ridges received enough water, which enabled these plants to perform better compared to the plants grown in furrows. NaCl treatments occasionally affected root growth but had low influence on GS accumulation.

Despite the fact that water management using different soil designs was not adequate to enhance GS in horseradish tubers, it causes a clear increase in biomass of the roots system. Therefore, these soil designs can be used to improve horseradish yield quantitatively.

Simultaneous occurrence of multiple stresses affected GABA accumulation differently. Therefore, no clear pattern of GABA accumulation could be deduced.

The experiments carried out on the deliberate application of exogenous factors and their effects on GS accumulation in horseradish plants, indicated that GS concentrations can be modulated and enhanced. However, the degree of control of the culture system plays a major role for the expected effect of these factors on GS accumulation.

General remarks of the effects of abiotic factors on glucosinolate accumulation in horseradish mature plants

The experiments conducted with horseradish plants as an experimental system revealed that there might be a stress induced enhancement of GS concentrations, however mostly due to the general fact that stressful conditions are accompanied by decreases in biomass, which might concentrate the prevailing GS in stressed plants. Additionally, the central question concerning the enhanced biosynthesis due to over reduced status under stress conditions could not be answered unequivocally. However, the SA experiment elucidated the importance of other physiological processes and their possible effects on GS accumulation. One of these aspects is the role of GS translocation. Thus, horseradish does represent an optimal experimental system for the elucidation of basic scientific issues on stress, and their impact on GS accumulation. On the other hand, horseradish is an ideal system to investigate other processes that might affect GS accumulation. Also with respect to applied issues, horseradish represents a very good model plant.

Apart from the quality aspect, the data demonstrate that horseradish seems to be a very good system to analyze both remobilization and translocation of GS. For example, senescing leaves are quite appropriate to study the phenomenon of GS mobilization. Alternatively, they can be used to study GS translocation. In order to prove translocation, biosynthetic capability for GS should be determined in leaves and roots. Furthermore, differentiation between GS remobilization and translocation is needed.

Due to the humid climate in Germany and the high WHC of the soil used to grow plants in the field, future experiments should focus on investigating drought effects in other types of soil characterized by low WHC, such as sandy soils. Alternatively, this approach could be used in countries with less humid climate to investigate the applicability of enhancing the contents of secondary plant products.

Although GS are in the center of focus in this work, myrosinase is a key component of GS containing plants, which is responsible for its degradation. Sulfur deficiency is known to

enhance myrosinase activaty (Bones and Rossiter, 1996), and recent reports suggested that myrosinase activity was also increased in response to abiotic stresses (Pang et al., 2012). Therefore, the effects of water stress, especially drought on myrosinase activity was also analyzed.

4.4. Effects of drought on myrosinase activity

Abiotic stresses have been suggested to enhance myrosinase activity (Pang et al., 2012). Accordingly, a brief investigation of water stress effects on myrosinase should be introduced in this work. Enhanced enzyme activity might increase taste perception during mastication of GS containing plants.

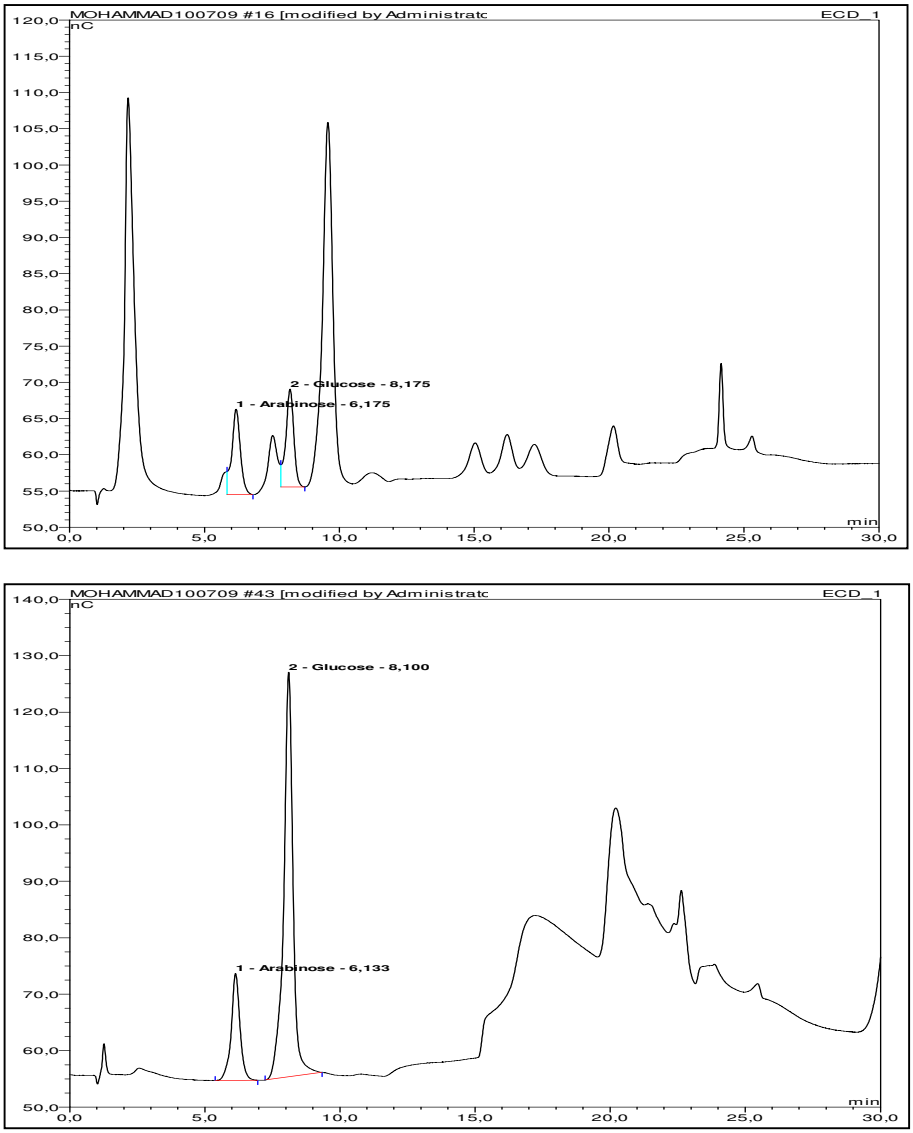


Figure 62: Soluble sugars standard (A), and enzyme assay (B).

For accurate determination of enzyme activity, a reliable method is needed. Numerous methods are available to measure myrosinase activity. However, many of these methods were affected by the presence of ascorbic acid, especially in the case of myrosinase inactivity in the absence of ascorbic acid. Additionally, ascorbic acid interfered with most of the standard

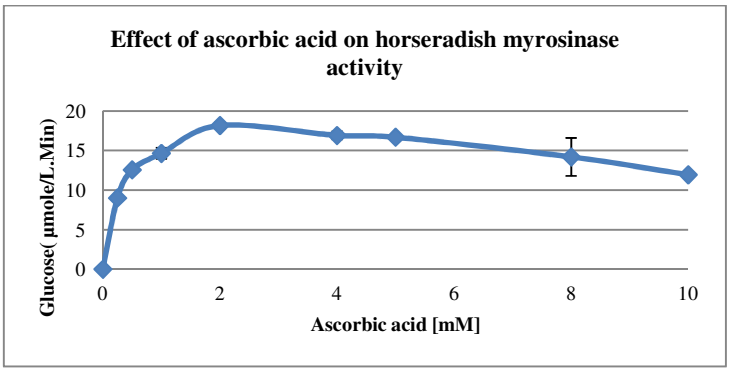
assays used for myrosinase activity determination. (Kleinwächter and Selmar, 2004). Kleinwächter and Selmar (2004) reviewed these methods and developed a reliable method for myrosinase activity determination. However, this method needs a considerable effort for the measurement of enzyme activity. Therefore, an appropriate method, allowing improved myrosinase activity determination should to be adopted.

For this purpose, liberated glucose as primary reaction product and arabinose as internal standard (ISG) were directly determined using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which is considered to be one of the most sensitive means to measure soluble sugars in natural samples (Grey et al., 2009). Figure 62 shows chromatograms of a mixed soluble sugar standard (A) and a typical enzyme assay (B).

Using the newly developed method, basic characteristics of horseradish myrosinase had been determined. Several basic parameters affecting myrosinase activity, such as ascorbic acid concentration, substrate concentration, pH and temperature, were investigated. Obtained results show that myrosinase activity is highly sensitive to ascorbic acid at low concentrations, and optimum ascorbic acid concentration is 2 mM. However, at high concentrations, ascorbic acid inhibits enzyme activity (Figure 63, A). Unfortunately, further attempts to determine ascorbic acid concentrations capable of completely inhibiting myrosinase activity was not successful due to the sensitivity of the device to ascorbic acid.

Myrosinase activity increased in response to increasing substrate (sinigrin) concentrations until 2 mM, after that the activity was nearly constant (Figure 63, B). Moreover, myrosinase was highly sensitive to pH and temperature. The optimum pH was 5.5 (Figure 63, C), while optimum temperature for enzyme activity was found to be 40 ^{o}C. Finally, Vmax and Km value were determined from Lineweaver-Burk plot to be 10.7 µmole/min and 0.31 mM, respectively (Figure 63).

A.



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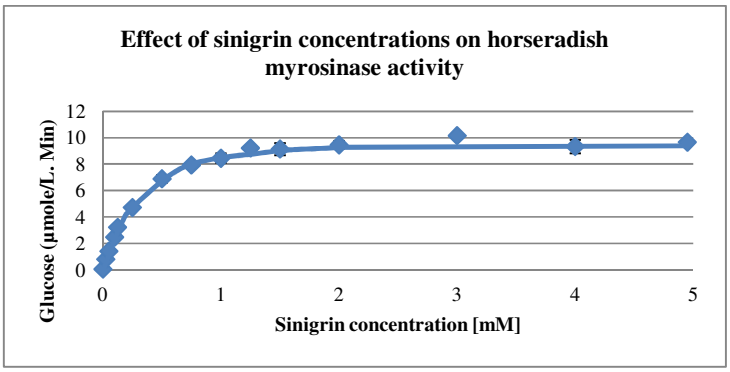
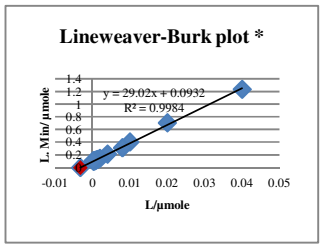
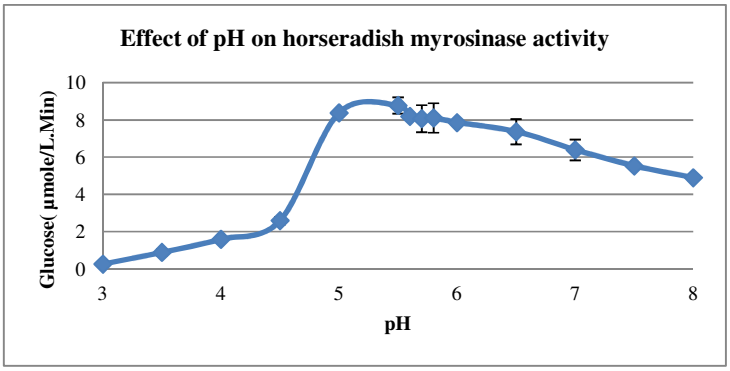




C.



D.

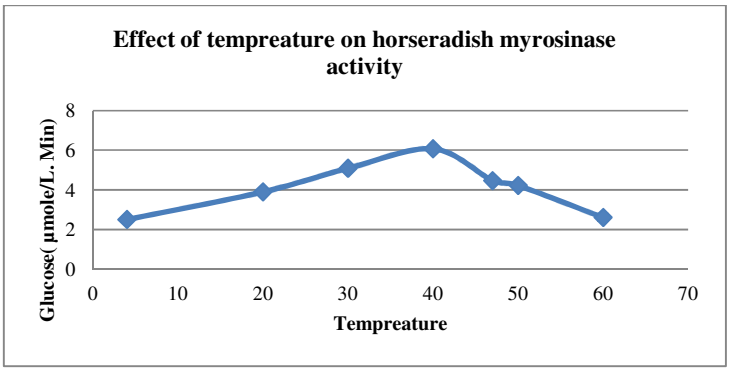


Figure 63: Effects of different parameters on horseradish soluble myrosinase activity. Ascorbic acid (A), substrate (sinigrin) (B), pH (C) and temperature (D). Decrease in enzyme activity is due to enzyme dilution for optimal activity measurement by HPAEC. * Full size of Linweaver-Burk plot is provided in the appendix (Figure A10).

As standard conditions for the myrosinase activity determination the following conditions had been set: 2 mM ascorbic acid, pH 5.5, 2 mM sinigrin (substrate) and 40 °C. Using this standard assay, samples for well-watered control and water stressed plants (drought as well as excess water) had been analyzed. Unfortunately, no correlation between different treatments and the enzyme activity was found (Figure 64).

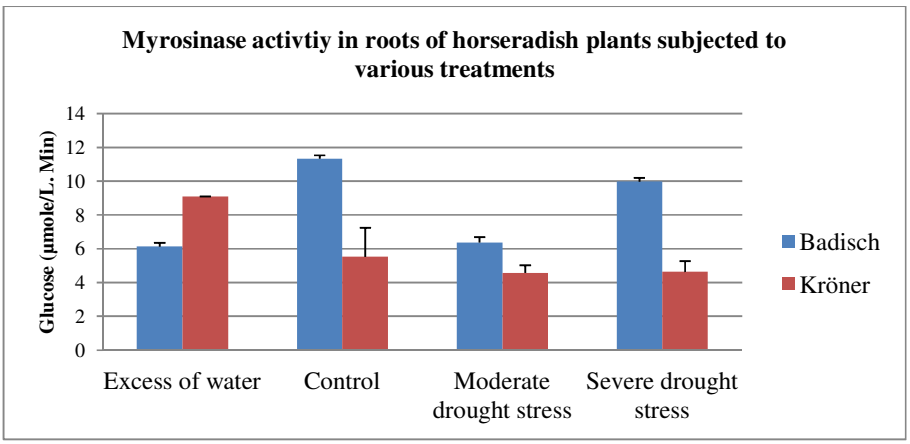


Figure 64: Effect of various treatments on soluble myrosinase activity in roots of horseradish plants from Badisch and Kröner variety.

Discussion:

Soluble myrosinase isolated from different organs and species showed a considerable degree of variation in its activity in response to reaction conditions. For instance, optimum temperature varied from 37 °C in *C. abyssinica* leaves and seeds to 75 °C in *B. napus* seeds, and pH optimum varied from 4.2 in *S. alba* to 9.0 in *C. abyssinica*. Moreover, ascorbic acid also plays an important role for myrosinase activation (Martin et al., 2008; Bernardi et al., 2003; Kleinwächter and Selmar, 2004). Myrosinases are also known to accept a wide range of GS as substrate or to be highly specific, but they have no activity toward any other naturally occurring substrate (Halkier and Gershenzon, 2006).

The ascorbic acid optimum concentration (2 mM) for horseradish myrosinase determined in this study was also found for myrosinase activation in *T. majus* (Kleinwächter and Selmar, 2004). Moreover, horseradish root tissues were reported to contain 2 mM ascorbic acid (Bones and Rossiter, 1996). Li and Kushad. (2005) confirmed the effect of ascorbic acid on myrosinase activity, but the highest concentration they investigated was 0.5 mM, therefore no comparison can be made with their finding. In accordance to the results obtained here,

inhibition of myrosinase activity at higher ascorbic acid concentrations (higher than 2 mM) was frequently reported (Figure 63, A).

Obtained Vmax and Km values are different from that reported earlier for horseradish roots of 0.625 µmole/min and 0.128 mM, respectively (Li and Kushad, 2005). Comparisons with myrosinase activity from other species, such as *T. majus*, using different substrate was difficult (Kleinwächter and Selmar, 2004), since myrosinases from various species show different characteristics, and sensitivities to different GS (Bones and Rossiter, 1996; Martin et al., 2008). Nevertheless, obtained Km value, is within broad Km values reported for myrosinase obtained from different species (Shikita et al., 1999; Björkman et al., 1973)

Optimum pH (5.5) for horseradish myrosinase obtained here is quite similar to the previously reported optimum pH of 5.7 (Li and Kushad, 2005).

Temperature optimum obtained in these experiments (40 ^{o}C) is also different from previously reported optimum temperature of 45 ^{o}C (Li and Kushad, 2004).

No effects of drought stress on myrosinase activity could be detected. Thus, with respect to the effects of abiotic factors on myrosinase activity, the only reported case was an activity enhancement caused by limitation of sulfur (Bones and Rossiter, 1996) A recent publication on myrosinase activation upon NaCl treatment has to be questioned, since the observed effects are rather due to reduced supply of sulfur, than to a direct effect of NaCl. Moreover, in this study the ascorbic acid effect on myrosinase activation was not considered (Zaghdoud et al., 2012; Pang et al., 2012).

In conclusion, our results show that exogenous abiotic factors can cause an enhancement in GS concentrations in horseradish plants, especially under controlled conditions. However, many factors need to be optimized before abiotic factors can deliberately applied for improving horseradish tubers quality for commercial purposes. Myrosinase activity is not affected by drought stress. However, further investigations of the effects of abiotic factors on myrosinase activity are needed to elucidate the various factors influencing and controlling the enzyme activity.

5. Summary and perspectives

Glucosinolates (GS) represent an interesting and important class of secondary plant products. Their biosynthesis is affected by various plant interactions with their surroundings. Moreover, glucosinolates reveal a great importance for human health. GS biosynthesis has been reported to be induced in response to a biotic stresses, but only few sound investigations on this issue are available. Accordingly, GS accumulation in response to exogenous abiotic factors was studied in horseradish as model plant.

The concentration of horseradish major GS, i.e., sinigrin (SI) and gluconasturtiin (GN), were efficiently analyzed by ion pair chromatography (IPC). This method enables the analysis of intact GS and eliminated the need for long extraction procedures. Changes in SI and GN concentrations were used to study the impact of exogenous abiotic factors on GS accumulation in mature and *in vitro* horseradish plants.

Various exogenous factors impacting GS biosynthesis have been investigated in horseradish. The effect of sulfur supply on GS accumulation was studied in *in vitro* plants, whereas drought stress and salinity had been analyzed in field experiments as well as using *in vitro* plants. In the later case, plants were exposed to media containing different amounts of PEG in order to diminish water availability. Phytohormones relevant for stress signaling, such as salicylic acid and abscisic acid, had been applied to investigate their role in GS metabolism in mature as well as in *in vitro* plants.

GS accumulation is highly sensitive to sulfur supply; sulfur reduction in media to 0.23 mM caused severe reductions in GS concentrations in all plant parts. Sulfur enhancement to 8.3 mM resulted in corresponding increases in GS concentrations. A further increase of sulfur concentrations (from 8.3 mM to 21.5 mM) revealed only slight changes in GS concentrations. The development of the root system of i*n vitro* plants did not affect GS accumulation markedly; although root system formation was improved due to an increase of naphthalene acetic acid (NAA) concentrations. Thus, sulfur supply is the major factor affecting GS accumulation, whereas the shape of the root system has only little effect on GS concentrations in horseradish *in vitro* plants.

Drought stress induced by PEG treatments caused a massive increase in GS concentrations. However, drought stressed plants also revealed a massive decrease in biomass. Therefore, GS

contents on a whole plant basis are lower in plants cultured under drought stress, i.e., on media containing PEG, than in control plants. Thus, no impact on biosynthesis due to drought stress was detected.

Similar extent of GS accumulation was also observed in *in vitro* plants subjected to moderate salinity stress by NaCl application. The plants exposed to 25 mM NaCl accumulated higher GS concentrations compared to control plants. On the other hand, stressed plants, which were cultured on media containing 100 mM NaCl revealed lower GS concentrations than control plants. Although salinity stress has less effect on biomass compared to drought stress, the total content of GS was markedly reduced in comparison to control plants. Thus, also for salinity, there is no conclusive evidence on increased GS biosynthesis.

Plants cultured on media containing abscisic acid (ABA, 5 and 10 mg/L) concentrations revealed higher GS concentrations than control plants. However, due to a huge decrease in biomass of ABA treated plants, control plants contained higher total GS contents than the plants subjected to ABA treatments. Accordingly, conclusive deductions on the effect of ABA treatments on GS biosynthesis rate are not possible.

Horseradish plants grown under rain shelter and subjected to SA treatments showed no differences in GS concentrations in roots. However, due to their larger roots, control plants accumulated higher amounts of GS. In contrast, leaves of horseradish plants treated with 5 mM SA accumulated higher GS concentrations compared to control plants, while leaves of plants treated with 1 mM SA revealed lower GS concentrations. The impact of this growth regulator on GS content is similar to that observed for ABA. This indicates that both molecules may affect GS biosynthesis indirectly and probably by analogous mechanisms.

Drought caused an increase in GS concentrations in mature horseradish plants grown under rain shelter. Moderate drought induced by water limitation entailed a significant increase in GS concentrations in all three varieties tested. However, control plants developed larger roots. Consequently, the total content on a whole plant basis was nearly the same; although differences in GS concentrations between control and drought treatments occurred. Thus, no conclusive evidence on enhanced GS biosynthesis could be concluded.

The field experiment revealed unexpected results. Plants that were grown under putative drought stress conditions, i.e., grown in ridges, where they received less water than the well watered plants cultivated in the furrows, developed much better and revealed lower GS

concentrations. Obviously, due to massive rainfall in summer, plants grown in ridges received appropriate water supply. On the other hand, plants grown in furrows received too much water. Horseradish plants require deep, well aerated soils. Therefore, compact and wet soils in furrows were unfavorable for horseradish plants growth. This resulted in retarded growth but higher GS concentrations in these plants compared to plants grown in ridges. NaCl treatments affected horseradish plants differently, depending on the water amounts they received. NaCl treatments slightly affected vegetative growth of plants grown in ridges, but have no effect on GS concentrations.

GABA concentrations used as stress indicator increased in response to drought in *in vitro* and rainshelter experiments. The patterns of GABA accumulation were similar to that of GS concentrations. Moreover, GABA concentrations were also increased in ABA and SA treated plants, but in different patterns. In contrast, GABA concentrations decreased in response to salinity. In the field experiment no clear response of GABA accumulation could be observed. Thus, GABA concentrations should not be used in future attempts as single stress indicator to estimate the extent of stress effects.

A reliable method for myrosinase activity determination was developed. The new procedure enables a much easier activity estimation of soluble myrosinase by direct determination of the liberated glucose. Moreover, this method is not affected by ascorbic acid, which generally intervenes with many previously reported methods for myrosinase activity determination. Myrosinase activity did not reveal a clear pattern in response to drought stress.

Basic physiological parameters, such as biomass and dry weight share, were markedly affected by abiotic stress, especially in *in vitro* experiments. Thus, these parameters must be considered in future investigations on the impact of abiotic stress on GS biosynthesis. Plants suffering from abiotic stress generally revealed lower biomass. Thus, it is argued that the observed high concentrations of secondary metabolites in stressed plants are resulting from a concentration effect due to small biomass. Accordingly, the total content, which considers the biomass, should be included for reliable estimation of actual stress effect on secondary metabolite biosynthesis. Only higher total contents of secondary metabolites in stressed plants compared to normally grown plants could prove actual enhancement in biosynthesis.

The enhancement of GS concentrations is of enormous importance to food quality and human health. Thus, an improvement of the quality of commercial crops by increasing their

concentrations of GS should be realised. Moreover, due to the simple setup by using ridges and furrows, such approach is environmentally friendly. However, optimization of GS biosynthesis by influencing plant stress physiology is not trivial, since the precise degree of stress causing positive effects has to be determined. Moreover, managing numerous factors that might interfere with GS biosynthesis under field conditions is quite challenging. Finally, reductions in yield, which are concomitant with most stress conditions, still a major drawback of manipulating plant physiology toward enhanced production of useful metabolites in plants.

The results on the accumulation of GS outline that horseradish could not be only a model plant for applied aspects of GS accumulation but also for basic aspects of GS biosynthesis physiology, i.e., translocation or *in vivo* mobilization. Accordingly, horseradish resembles an ideal experimental plant for both applied commercial aspects and basic biochemical issues of GS metabolism.

Although data obtained in this work did not yield a conclusive evidence of enhanced GS biosynthesis due to abiotic factors, there are various interesting observations concerning the experiments conducted with *in vitro* plants that should be addressed in the future. In this context special emphasis should be put on GS mobilization, an issue only little information is known about: Sulfur starvation experiments could be a suitable tool to investigate this topic in the future. Signaling molecules such as ABA and SA affect GS biosynthesis in a different manner. Therefore, they could be an appropriate supplementation to stress application such as drought and salinity. ABA and possibly other molecules could be used to investigate the role of stress signaling networks on GS metabolism. In general, light intensity can be used to modulate the stress in *in vitro* experiments.

Also the experiments with mature plants highlighted the importance of GS mobilization and translocation. The phenomenon of leaf senescence provides very suitable experimental conditions for simultaneous investigations of these not well understood phenomenons of GS. The fact that exogenous SA application caused increases in GS concentrations in leaves but not in roots of the same plant provides another possibility to investigate GS translocation. This should be conducted in parallel with molecular studies of GS biosynthesis in various plant organs.

6. References

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Table A1: Sulfur content in the field soil.

Field design	**Sulfur (mg/kg)**
Furrow	24
Ridge	14

Table A2: SI, GN and total GS concentrations of leaves, stems and roots of horseradish *in vitro* plants cultured on MS media supplemented with 0.5 mg/L NAA and (0.23, 1.7 (standard), 8.3 and 21.5) mM SO_4^{2-}, over a growth period of six weeks. Data represent average of two independent extractions, except for 21.5 mM SO_4^{2-} after six weeks of culture, were data represent the average of nine individual extractions and analysis of different plant parts (leaves, stems and roots) of nine different plants.

Treatment SO_4^{2-} [mM]	Leaves/ two weeks					Leaves/ four weeks					Leaves/ Six weeks				
	SI	StD	GN	StD	Total GS	SI	StD	GN	StD	Total GS	SI	StD	GN	StD	Total GS
0.23	14.81	0.12	0.00	0.00	14.81	6.46	0.15	0.00	0.00	6.46	2.41	0.64	0.00	0.00	2.41
1.7	26.37	0.25	0.38	0.03	26.76	25.92	1.44	0.15	0.22	26.07	22.26	1.99	0.42	0.09	22.68
8.3	38.53	0.89	0.49	0.08	39.03	40.20	2.06	0.00	0.00	40.20	39.56	5.62	0.45	0.04	40.01
21.5	41.58	1.74	0.00	0.00	41.58	40.13	0.83	0.00	0.00	40.13	40.27	3.70	0.16	0.21	40.44
Treatment SO_4^{2-} mM	Stems/ two weeks					Stems/ four weeks					Stems/ Six weeks				
	SI	StD	GN	StD	Total GS	SI	StD	GN	StD	Total GS	SI	StD	GN	StD	Total GS
0.23	10.99	2.90	0.74	0.32	11.74	6.80	0.06	0.27	0.38	7.08	1.91	0.54	0.20	0.18	2.11
1.7	27.12	0.93	0.88	0.11	28.01	24.70	0.66	1.05	0.05	25.75	24.54	2.09	1.46	0.39	26.00
8.3	31.48	0.65	0.95	0.22	32.43	33.54	1.24	0.66	0.03	34.21	31.05	4.84	0.97	0.29	32.02
21.5	34.05	1.28	0.40	0.57	34.45	39.84	0.35	0.71	0.04	40.56	36.28	3.59	0.82	0.12	37.10
Treatment SO_4^{2-} mM	Roots/ two weeks					Roots/ four weeks					Roots/ Six weeks				
	SI	StD	GN	StD	Total GS	SI	StD	GN	StD	Total GS	SI	StD	GN	StD	Total GS
0.23	0.00	0.00	0.92	0.23	0.92	0.00	0.00	0.45	0.23	0.45	0.32	0.55	0.43	0.26	0.75
1.7	1.93	0.15	2.60	0.05	4.53	0.60	0.07	1.61	0.05	2.21	1.65	0.27	2.10	0.67	3.75
8.3	1.63	0.92	1.12	0.19	2.75	0.64	0.10	1.99	0.00	2.63	2.57	1.10	1.66	0.48	4.23
21.5	1.64	0.23	1.59	0.03	3.23	1.45	0.07	1.80	0.04	3.25	0.59	1.19	1.25	1.66	1.84

Table A3: SI, GN and total GS concentrations in horseradish plants roots subjected to different treatments on dry matter basis. Differences in total GS concentrations were not statically significant at 0.05 degree of confidence according to student T test. Similar letter indicate significantly indifferent results between plants receiving the same NaCl amount. Data are average of double estimations of seven plants.

East variety		Glucosinolates [µmole/g d.w.]					
Soil design	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
Furrows	**0**	96.35	15.29	15.34	2.94	111.68a	12.79
Ridges	**0**	96.47	11.77	15.50	5.93	111.98a	15.51
Furrows	**25**	99.98	9.76	13.80	3.20	113.78a	9.96
Ridges	**25**	97.36	11.37	12.02	2.39	109.38a	9.68
Furrows	**50**	93.74	15.18	13.96	4.44	107.71a	12.44
Ridges	**50**	85.12	18.85	14.55	2.93	99.66a	19.84
Furrows	**100**	101.58	16.73	13.88	3.65	115.47a	15.07
Ridges	**100**	96.34	16.80	16.15	7.51	112.49a	21.24

Badisch variety		Glucosinolates [µmole/g d.w.]					
Soil design	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
Furrows	**0**	92.64	14.41	11.93	4.24	104.57a	15.74
Ridges	**0**	84.61	13.59	12.10	2.11	96.71a	13.06
Furrows	**25**	91.42	8.60	10.35	1.74	101.77a	7.77
Ridges	**25**	87.77	16.84	12.55	2.60	100.32a	16.36
Furrows	**50**	91.88	13.44	12.53	4.88	104.41a	11.76
Ridges	**50**	94.81	5.16	12.22	2.21	107.02a	6.27
Furrows	**100**	90.65	13.87	12.54	1.46	103.19a	13.76
Ridges	**100**	88.82	8.50	11.26	1.51	100.08a	8.99

Kröner variety		Glucosinolates [µmole/g d.w.]					
Soil design	**NaCl [mM]**	**Sinigrin**	**StD**	**Gluconasturtiin**	**StD**	**Total glucosinolates**	**StD**
Furrows	**0**	105.17	17.09	12.16	2.69	117.33a	15.54
Ridges	**0**	91.55	10.35	10.15	0.92	101.70a	9.71
Furrows	**25**	103.54	7.42	11.3	1.27	114.84	7.20
Ridges	**25**	87.91	6.73	10.24	2.40	98.15b	5.11
Furrows	**50**	107.51	8.67	10.94	1.80	118.44a	8.46
Ridges	**50**	92.72	7.51	10.80	2.03	104.46b	7.06
Furrows	**100**	93.29	15.49	11.25	2.05	104.54a	13.85
Ridges	**100**	94.15	12.57	12.55	2.61	106.70a	10.63

Table A4: GABA concentrations in horseradish roots of plants subjected to different treatments in the field from East (A), Badisch (B) and Kröner (C) varieties. Data are average of double estimations.

A.

Soil design	NaCl [mM]	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Furrows	**0**	29.46	1.56	9.52
Ridges	**0**	32.75	2.02	9.79
Furrows	**25**	34.72	7.86	10.83
Ridges	**25**	26.07	1.36	8.65
Furrows	**50**	48.85	1.96	16.61
Ridges	**50**	39.03	1.27	12.86
Furrows	**100**	31.02	4.46	10.09
Ridges	**100**	45.52	4.85	13.86

B.

Soil design	NaCl [mM]	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Furrows	**0**	26.80	0.37	8.98
Ridges	**0**	20.58	1.71	6.84
Furrows	**25**	21.74	0.09	7.34
Ridges	**25**	25.87	4.03	8.14
Furrows	**50**	17.20	1.76	5.81
Ridges	**50**	33.25	6.12	10.71
Furrows	**100**	22.04	2.38	7.45
Ridges	**100**	22.43	0.87	7.38

C.

Soil design	NaCl [mM]	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Furrows	**0**	34.13	0.33	11.37
Ridges	**0**	28.33	1.52	9.38
Furrows	**25**	17.20	1.52	5.67
Ridges	**25**	25.81	0.43	8.38
Furrows	**50**	18.69	4.55	6.27
Ridges	**50**	15.68	0.92	4.97
Furrows	**100**	28.49	0.62	9.40
Ridges	**100**	30.41	1.17	9.99

Table A5: SI, GN and total GS concentrations in plants from Badisch and Kröner varieties on dry weight basis. Data are average of duplicates.

Variety	Soil design	Glucosinolates [µmole/g d.w.]				
		Sinigrin	StD	Gluconasturtiin	StD	Total glucosinolates
Badisch	Furrows	106.20	6.55	12.02	0.57	118.22
Badisch	Ridges	81.61	0.47	9.49	0.20	91.11
Kröner	Furrows	100.69	3.36	11.73	0.42	112.43
Kröner	Ridges	76.89	1.31	9.68	0.20	86.57

Table A6: GABA concentrations in horseradish roots of plants subjected to different treatments. Data resembles double estimations.

Variety	Soil design	GABA [µg/g d.w.]	StD	GABA [µg/g f.w.]
Badisch	Furrows	28.32	1.21	9.70
Badisch	Ridges	38.32	2.55	13.26
Kröner	Furrows	30.58	0.29	10.40
Kröner	Ridges	25.89	2.77	8.77

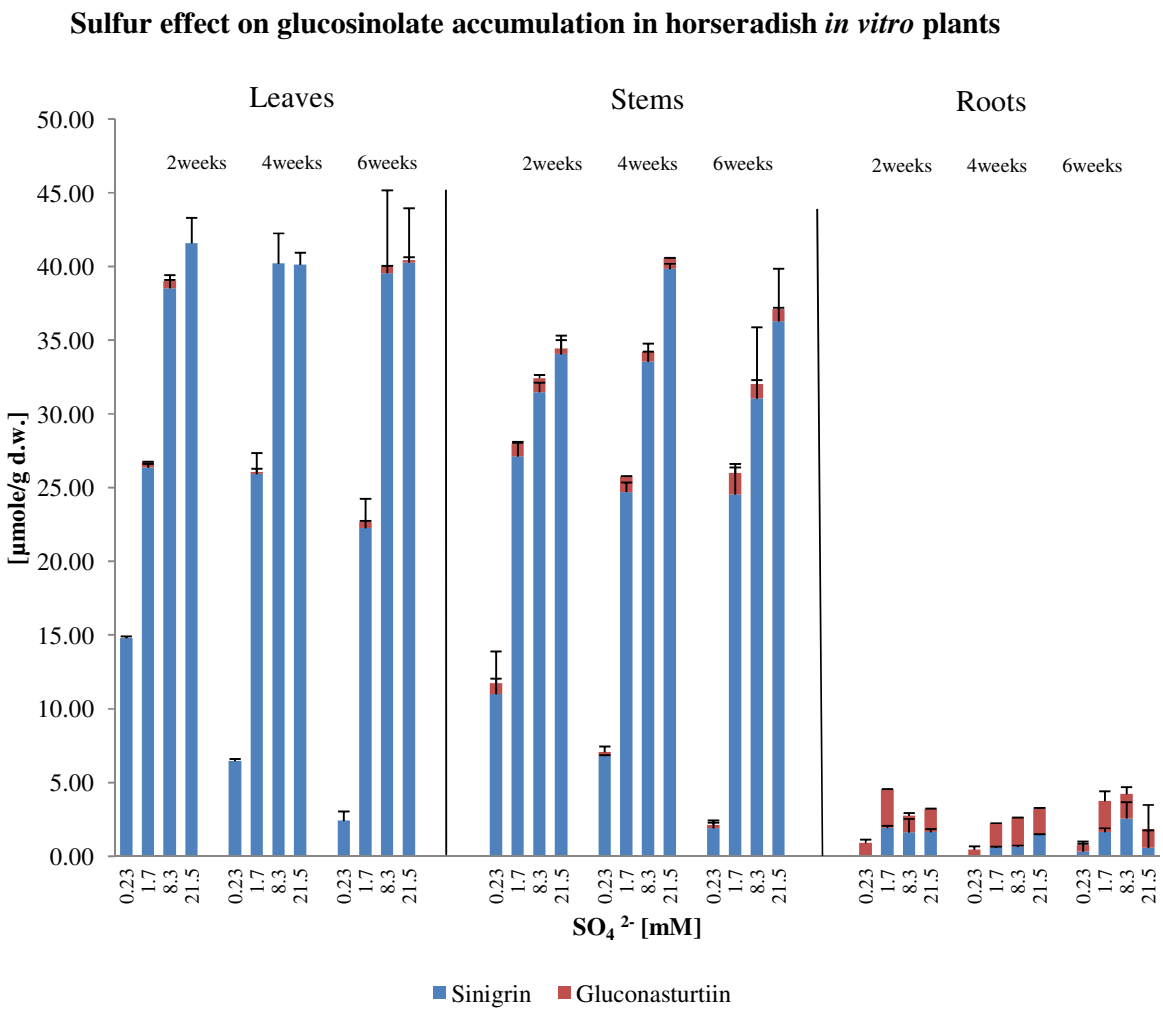
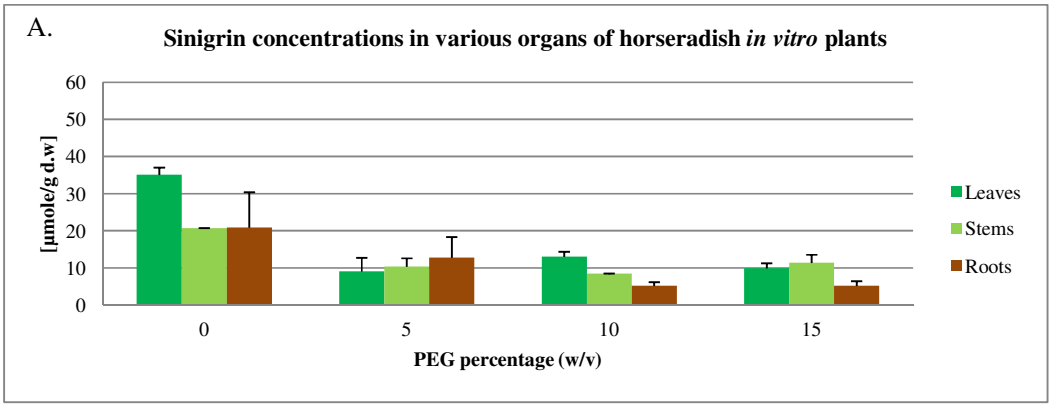


Figure A1: SI, GN and total GS concentrations of leaves, stems and roots of horseradish *in vitro* plants cultured on MS media supplemented with 0.5 mg/L NAA and (0.23, 1.7 (standard), 8.3 and 21.5) mM SO_4^{2-}, over a growth period of six weeks. Data represent average values of two independent extractions, except for 21.5 mM SO_4^{2-} after six weeks of culture, were data represent the average of nine individual extractions and analysis of different plant parts (leaves, stems and roots) of nine different plants. Error bars resemble StD.



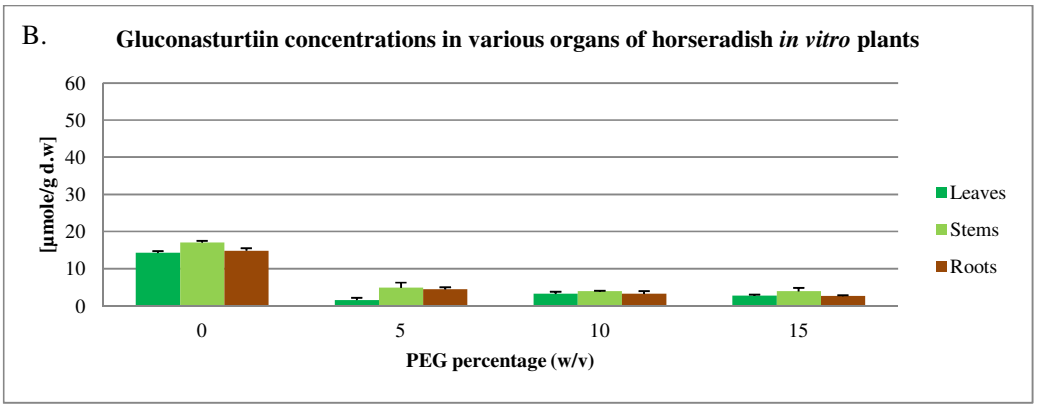


Figure A2: SI (A) and GN (B) concentrations on dry weight basis in horseradish *in vitro* plants organs after four weeks of PEG treatments. Data are duplicates of two individual extractions. Error bars resemble StD.

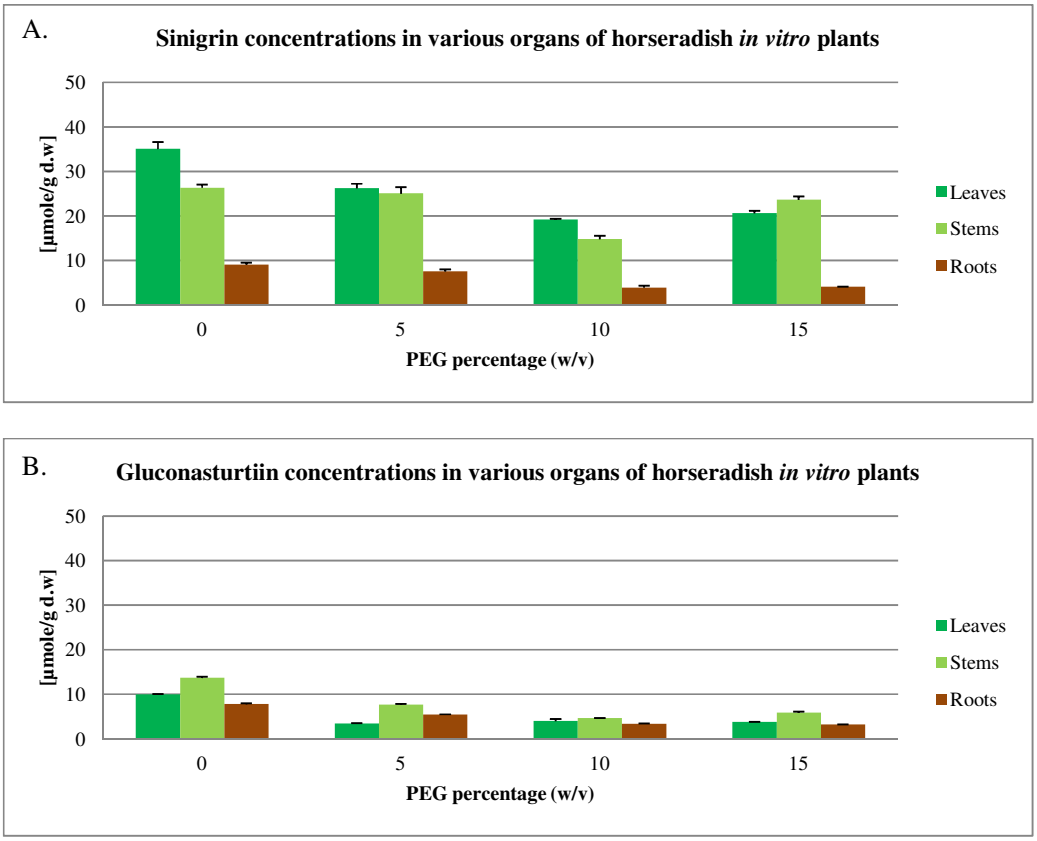


Figure A3: SI (A) and GN (B) concentrations on dry weight basis in horseradish *in vitro* plants organs after six weeks of PEG treatments. Data are duplicates of two individual extractions. Error bars resemble StD.

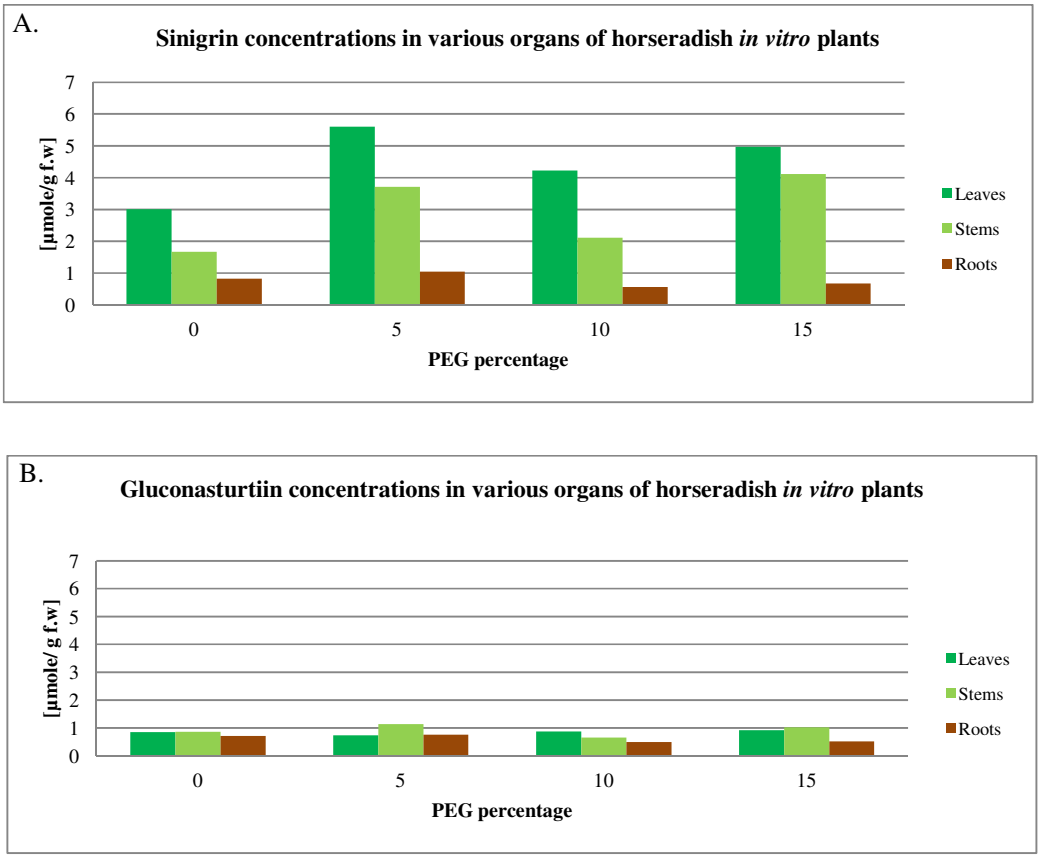


Figure A4: SI (A) and GN (B) concentrations on fresh weight basis in horseradish *in vitro* plants organs after six weeks of PEG treatments.

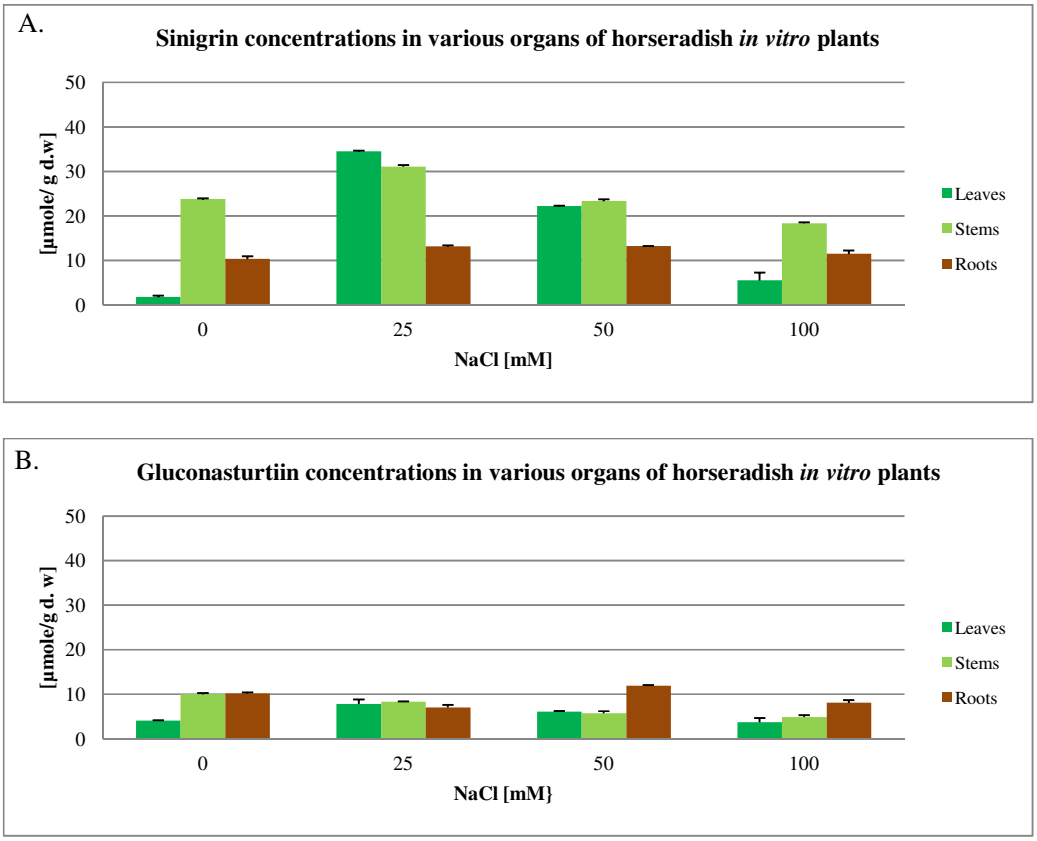


Figure A5: SI (A) and GN (B) concentrations on dry weight basis in horseradish *in vitro* plants organs after six weeks of NaCl treatments. Data are duplicates of two individual extractions. Error bars resemble StD.

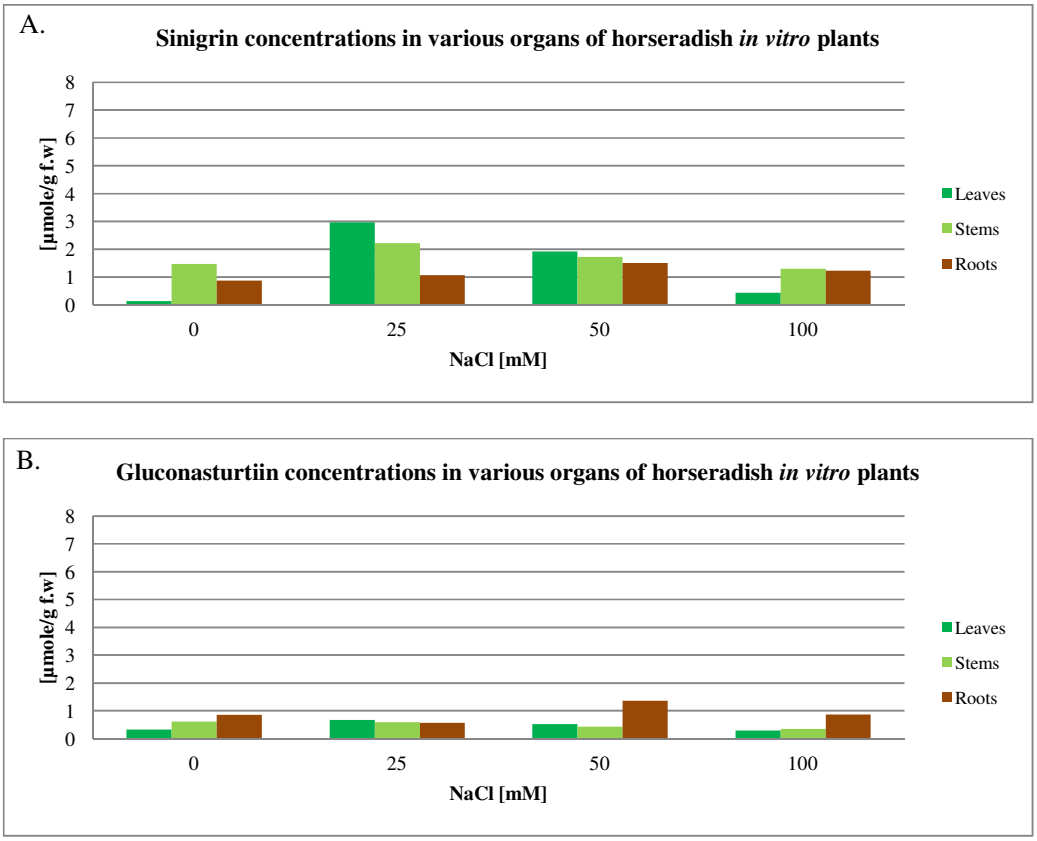


Figure A6: SI (A) and GN (B) concentrations on fresh weight basis in horseradish *in vitro* plants organs after six weeks of NaCl treatments.

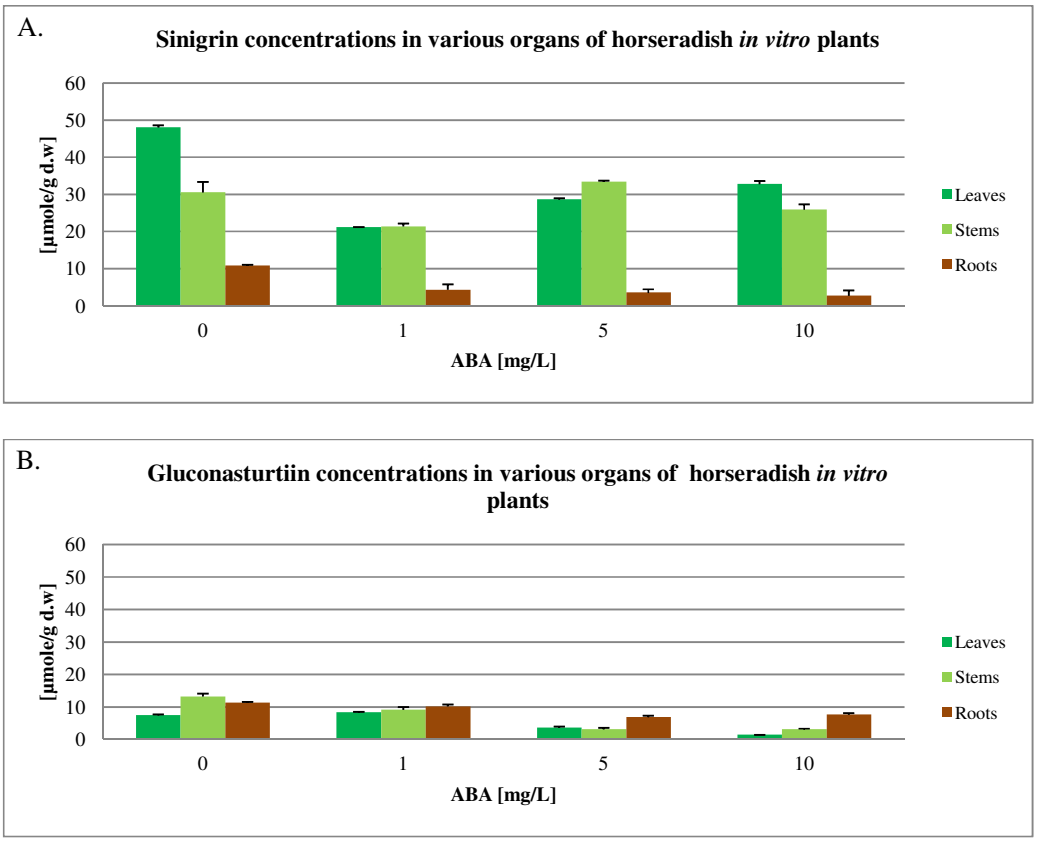


Figure A7: SI (A) and GN (B) concentrations on dry weight basis in horseradish *in vitro* plants organs after six weeks of ABA treatments. Data are average of duplicates. Error bars resemble StD.

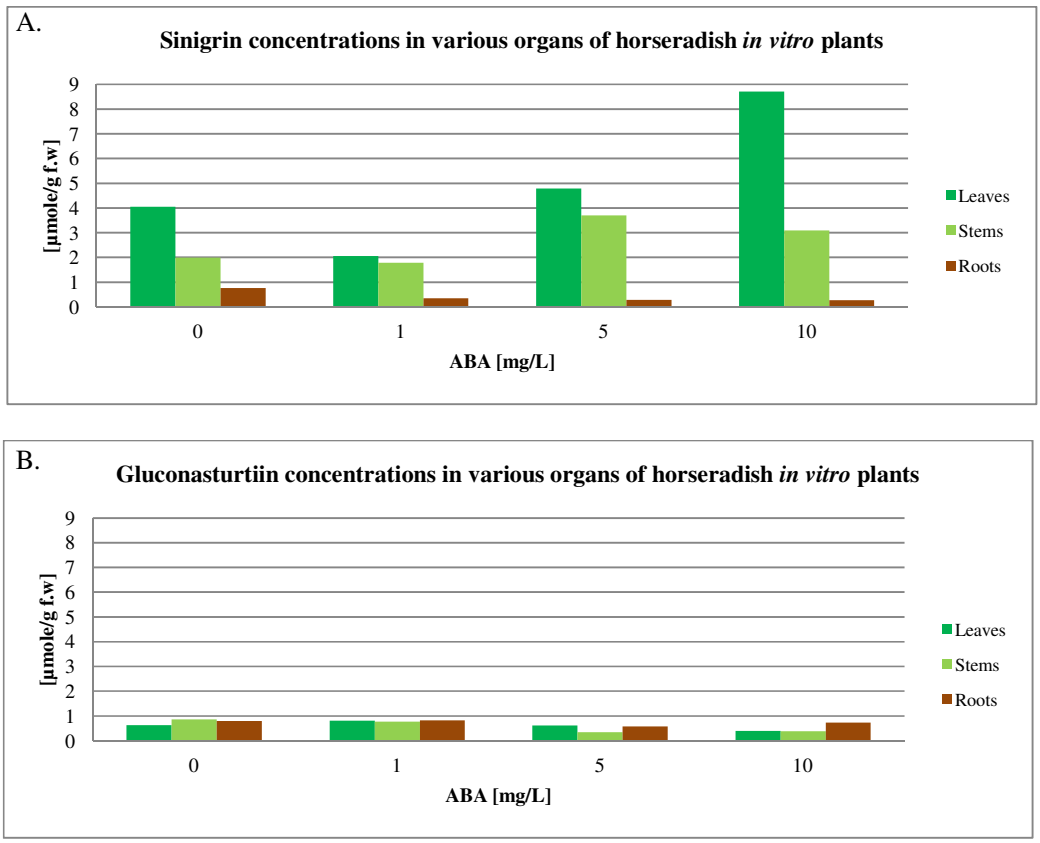


Figure A8: SI (A) and GN (B) concentrations on fresh weight basis in horseradish *in vitro* plants organs after six weeks of ABA treatments.

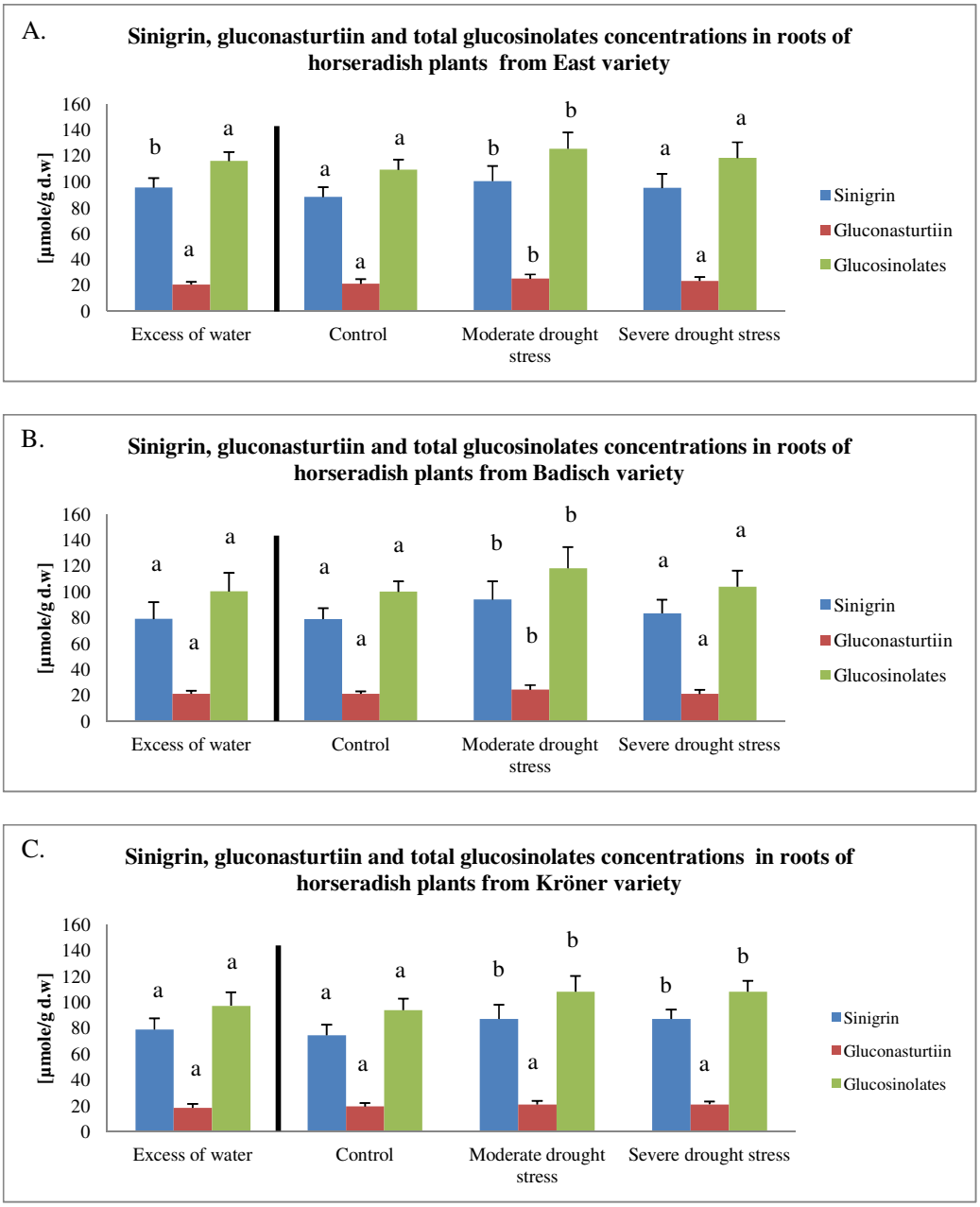


Figure A9: Effect of various treatments on SI, GN and total GS concentrations on dry weight basis in horseradish roots of plants from East (A), Badish (C) and Kröner (E) variety, respectively. Data are average of duplicates of ten individual plants. Error bars resemble StD. Different letters indicate significant difference compared to control plants, when data were analyzed according to student T test at 0.05 degrees of confidence.

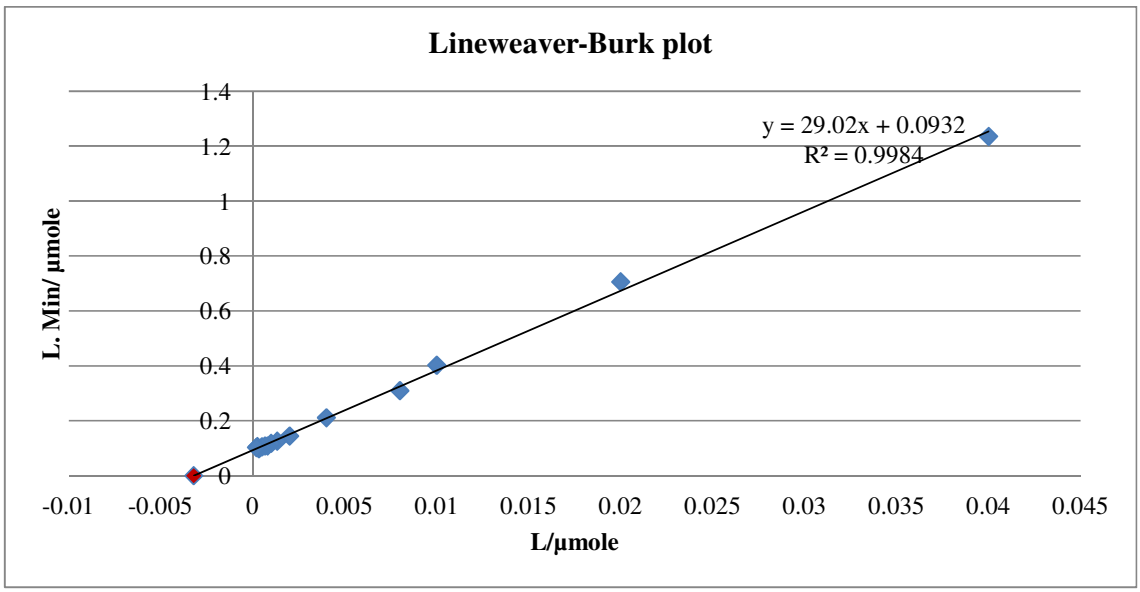


Figure A10: Lineweaver-Burk plot.

Chemicals and reagents

1-naphthelen acetic acid	≥ 95%, Sigma (Steinheim, Germany) (Plant cell culture tested)
2, 4-dichlorophenoxyacetic acid	≥ 98%, Sigma (Steinheim, Germany) (Plant cell culture tested)
2-phenylethyl glucosinolates, potassium salt (gluconasturtiin)	C_2 Bioengineering ApS, (Copenhagen, Denmark)
2-propenyl glucosinolates, potassium salt (sinigrin)	Carl-Roth (Karlsruhe, Germany)
(±)-abscisic acid	≥ 98.5 %, Sigma (Germany) (Plant cell culture tested)
Benzyl adenine	Sigma (Steinheim, Germany) (Plant cell culture tested)
4-hydroxy benzylglucosinolates (sinalbin)	C_2 Bioengineering ApS, (Copenhagen, Denmark)
o-phynelphyldehyde	Fluka (Steinheim, Germany)
Salicylic acid	Sigma (Steinheim, Germany) (Plant cell culture tested)
Gamma aminobyturic acid	≥ 99 %, Sigma (Steinheim, Germany)
D-(-)-arabinose	HPLC, ≥ 99 %, Fluka (Steinheim, Germany)

D-(+)-glucose	HPLC, ≥ 99,5 %, Fluka (Steinheim, Germany)
Tetrabutylammonium hydrogen sulfate	HPLC, ≥ 98 %, J.T.Baker (Phillipsburg, Germany)
NaOH (50%)	Fluka (Steinheim, Germany) Eleuent for ion chromatography
NaCl	≥99.9% , Carl-Roth (Karlsruhe, Germany)
Polyethelyne glycol (PEG)	MW: 20 000, Serva (Heidelberg, Germany)